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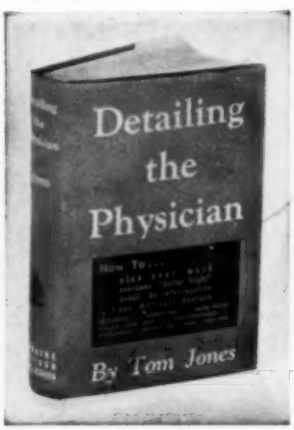

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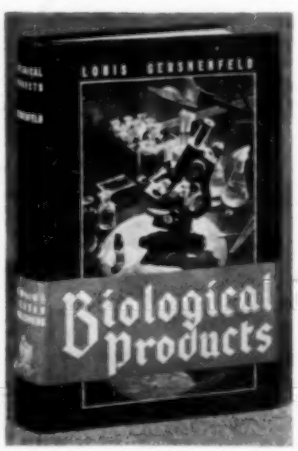

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Scientific Edition

JOURNAL OF THE AMERICAN PHARMACEUTICAL ASSOCIATION

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Volume XXX | *OCTOBER, 1941* | Number 10 Consecutive No. 19

The Biologic and Colorimetric Assay of Ergonovine in Ergot and Its Fluid Extract*

By C. E. Powell, O. W. Reagan, Asa Stevens and Edward E. Swanson†

Ergonovine is the new water-soluble base of ergot, the existence of which was first suspected by Moir (1) and clearly demonstrated by Davis, Adair, Rogers, Kharasch and Legault (2). This alkaloid, different from that of the water-insoluble members, ergotoxine and ergotamine, has a prompt oxytocic action when given by mouth as shown by Swanson and Hargreaves (3) in postpartum dogs. Ergonovine was isolated in a pure form, independently, by Kharasch and Legault (4), Dudley and Moir (5), Thompson (6) and Stoll and Burkhardt (7). The pharmacological action of the base was reported by Davis, Adair, Chen and Swanson (8), Chen, Swanson, Kleiderer and Clowes (9) and Brown and Dale (10). Ergonovine is now extensively used in medical practice as a uterine stimulant.

The present paper is concerned with the chemical separation and physiological assay of ergonovine in the crude ergot drug and fluid extract of ergot U. S. P. Previously, Swanson, Hargreaves and Chen (11) compared the various assay methods of pure ergonovine, and concluded that, in all respects, the isolated rabbit's uterus method gave most reliable figures. The oxytocic action of the isolated rabbit's uterus typifies ergonovine and eliminates the ergotoxine-like alkaloids, and the prolonged rhythmic contractions distinguish it from histamine and tyramine, both of which cause brief responses. The contractions of the mature virgin organ (isolated rabbit uterus) are proportional to the amounts supplied, which fact may be utilized for the quantitative evaluation of the base (ergonovine), just as the isolated guinea-pig uterus has been used for the assay of posterior pituitary extracts. This method (isolated rabbit's uterus), however, is of little value if the ergonovine is contaminated with the water-insoluble alkaloids (ergotoxine-like). In previous communications (8, 11), it has already been emphasized that the effects of ergonovine on the isolated rabbit's intestine and uterus are diminished or inhibited after the previous application of ergotamine or ergotoxine.

* Presented to the Scientific Section of the A. Ph. A., Detroit meeting, 1941.

† From the Lilly Research Laboratories, Eli Lilly and Company, Indianapolis.

Thus, because of this antagonism between ergonovine and ergotoxine, the Broom-Clark Method and the U. S. P. cock's comb Method are useless in differentiating the mixed alkaloids present in the crude ergot or its galenical preparation, the fluid extract U. S. P.

Hampshire and Page (12) described an assay process for ergot in which the water-soluble alkaloids are separated from the water-insoluble ones by shaking an ethereal extract with water and then determined colorimetrically. Schou and Bennekou (13) reported the distribution of ergonovine and ergotoxine between water and ether. Daglish and Wokes (14) have questioned the reliability of the Hampshire and Page method because of the difficulty of eliminating emulsions which appear when the ether extract is shaken with alkaline water. These authors found that acetone when used in extracting the drug in a mechanical shaker was a better solvent, without the formation of emulsions. In our hands, the water-soluble fraction (ergonovine) by the Hampshire and Page method could not be assayed biologically (isolated rabbit's uterus), because the solution still contained some of the water-insoluble alkaloids (ergotoxine-like). By further purification of this fraction (removal of the last traces of ergotoxine-like alkaloids), the colorimetric method still gave a much higher ergonovine content than the physiological method. This is probably due to the presence of the inactive water-soluble alkaloids (ergometrinine-like).

In view of the fact that Stoll (15), Kharasch and Legault (16), and we, in our own laboratory, found some samples of ergot completely devoid of ergonovine, and inasmuch as the known methods of assay are definitely unreliable, it became imperative either to modify the present methods or to devise a new one. One of the logical procedures, as outlined by Smith (17), would be the complete chemical separation of ergonovine from the ergotoxine-like alkaloids. This we have attempted to do. For the potency tests we have depended on the biological methods rather than the colorimetric method for reasons as previously stated. The ergonovine fraction was assayed by the isolated rabbit's uterus and the ergotoxine-like alkaloids were determined by the modified Broom-Clark Method (18). The physiologic test, however, was followed by the colorimetric method with the hope that an appropriate color filter could be found which would give readings similar to those of the biological assay. It was found that a light filter transmitting 6200 Å. gave the most concordant results.

EXPERIMENTAL

Method for Assaying the Crude Ergot Drug.—The directions for assaying the crude ergot drug may be given as follows:

"Place 12.5 Gm. of the defatted ground crude drug in a flask suitable for use in a mechanical shaker. Add 125 cc. of reagent acetone plus 2.5 cc. of 10 per cent ammonia, shake one hour, filter, take 100 cc. of the filtrate, which represents 10 Gm. of the drug, and evaporate under a current of air to approximately 25 cc. Add 10 cc. of distilled water plus enough tartaric acid to a pH of 3. Shake the acid-acetone-water solution with two 50-cc. portions of ether to remove excess fat and most of the coloring matter, and evaporate off the last traces of ether by vacuum distillation. Make the solution alkaline to a pH of 8 with sodium bicarbonate, allow to stand for 10 minutes, filter, and wash the container and filter paper with two 10-cc. portions of a 1 per cent solution of sodium bicarbonate. The precipitate on the filter paper represents most of the water-insoluble alkaloids (ergotoxine-like), and the filtrate contains the water-soluble alkaloid (ergonovine). Dissolve the precipitate on the filter paper in 10 cc. of a 2 per cent solution of tartaric acid, and assay by the Broom-Clark Method and colorimetrically for the ergotoxine-like alkaloids, with ergotoxine ethanesulfonate as the standard.

"In a separatory funnel, shake the filtrate (ergonovine) with two 50-cc. portions of carbon tetrachloride (10 minutes for each shaking) to remove the last traces of the ergotoxine-like alkaloids, run off the carbon tetrachloride, and remove all traces of carbon tetrachloride from the ergonovine solution by vacuum distillation (a little heat may be necessary). Add sodium chloride to saturation and shake with successive portions of ether until there is no blue color (*p*-dimethyl-aminobenzaldehyde reagent). Evaporate off the ether by air current in 10 cc. of distilled water made slightly acid with tartaric acid and assay biologically (isolated rabbit's uterus) and colorimetrically with pure ergonovine maleate as the standard."

The method described above was first tested on various mixtures of the pure alkaloids, ergotoxine ethanesulfonate and ergonovine maleate dissolved in the ammoniated acetone. As shown in Table I, both biologically and colorimetrically, an average of

more than 90 per cent of either alkaloid was accounted for. Thus, the results show that, working with solutions of the pure alkaloids, a quantitative determination of both alkaloids is possible.

With the crude ergot drug, the method was found to be useful in evaluating the ergonovine content. In the commercial isolation of ergonovine maleate, this method has been most helpful in the selection of good batches and in the yield of ergonovine. As shown in Table II, the thirty-seven lots of ergot contained from 2–20 mg. of ergonovine per 100 Gm. of crude ergot. The colorimetric method on the average gave a higher potency value for ergonovine than the oxytocic assay (isolated rabbit's uterus).

For the ergotoxine-like alkaloids in the drug and unlike that of the pure ergotoxine ethanesulfonate (Table I), the method was not so successful. The various solvents, gasoline or petroleum ether (used to defat the drug), and ether washings (to remove the coloring matter) also extracted some of the ergo-

Table I.—Mixtures of Pure Ergonovine Maleate and Pure Ergotoxine Ethanesulfonate

Experiment No.	Mixture of Pure Alkaloids		Colorimetric Method		Bioassay Methods	
	Ergotoxine Ethanesulfonate, mg. per 100 Cc.	Ergonovine Maleate, mg. per 100 Cc.	Ergotoxine Ethanesulfonate, mg. per 100 Cc.	Ergonovine Maleate, mg. per 100 Cc.	Broom-Clark Ergotoxine Ethanesulfonate, mg. per 100 Cc.	Oxytocic Ergonovine Maleate, mg. per 100 Cc.
1	50.0	10.0	46.0	9.4	46.0	9.0
2	50.0	20.0	49.0	19.4	52.0	20.0
3	50.0	25.0	50.0	24.5	53.0	22.0
4	50.0	15.0	47.5	14.4	49.5	14.6
5	30.0	10.0	24.1	9.0	29.0	8.5
6	48.0	10.0	48.0	9.4	54.0	9.5
7	50.0	5.6	40.0	5.0	47.0	4.4
8	50.0	7.0	50.0	6.0	42.0	6.8
9	50.0	13.0	45.0	12.0	43.0	9.7
10	50.0	10.0	50.0	10.0	43.0	9.0

Table II.—Crude Ergot

Lot No.	Colorimetric Method						Biological Methods					
		Ergotoxine Ethanesulfonate Equivalent, mg. per 100 Gm.				Ergonovine Maleate Equivalent, mg. per 100 Gm.		Boom-Clark Method Ergotoxine Ethanesulfonate Equivalent, mg. per 100 Gm.				Ergonovine Maleate Equivalent, mg. per 100 Gm.
	Defatted (Gasoline)	Ether Washings	Total Alkaloids	After Pptn. with $NaHCO_3$	CCl_4 Washings		Defatted (Gasoline)	Ether Washings	Before Pptn.	After Pptn. with $NaHCO_3$	CCl_4 Washings	
1	...	...	...	...	9.2	9.0	3.00	6.50	60.0	45.0	4.00	8.0
2	...	...	...	...	12.0	9.0	4.75	6.15	65.0	50.0	4.00	10.0
3	...	...	...	...	9.6	11.0	3.35	9.50	70.0	50.0	2.90	13.0
4	...	...	...	...	8.0	18.4	2.50	12.00	65.0	47.5	1.35	15.0
5	...	...	...	...	10.0	15.0	2.30	13.50	71.0	56.5	6.70	10.0
6	...	...	...	...	13.0	13.0	7.40	12.35	75.0	50.0	5.00	10.0
7	...	...	...	140.0	...	15.0	...	...	...	113.0	...	12.5
8	...	...	...	...	...	18.5	...	...	...	96.0	...	16.0
9	...	...	...	131.0	...	15.0	...	...	...	146.0	...	14.0
10	...	...	...	180.0	...	12.5	...	...	...	130.0	...	10.0
11	...	...	...	131.0	...	22.5	...	...	...	140.0	...	20.0
12	...	...	...	180.0	...	21.5	...	...	...	142.0	...	20.0
13	...	...	...	...	...	25.0	...	...	...	162.0	...	20.0
14	...	...	...	...	...	20.0	...	...	...	137.5	...	19.0
15	...	...	...	...	...	22.5	...	...	...	137.5	...	20.0
16	...	...	...	225.0	...	24.5	...	...	...	170.0	...	20.0
17	...	...	...	...	...	23.5	...	...	...	...	...	20.0
18	...	...	...	...	...	25.5	...	...	...	...	...	20.0
19	...	...	...	...	...	23.5	...	...	...	...	...	20.0
20	...	...	...	...	...	20.0	...	...	...	95.0	...	20.0
21	...	...	...	100.0	...	23.0	...	...	...	85.0	...	20.0
22	...	...	...	...	...	10.0	...	...	...	108.0	...	8.0
23	...	...	...	...	...	22.5	...	...	...	80.0	...	20.0
24	...	...	...	...	...	19.0	...	...	...	55.0	...	16.0
25	...	...	...	...	...	12.3	...	...	...	...	...	11.9
26	...	...	...	...	...	7.0	...	...	...	...	...	5.8
27	...	...	...	...	...	13.5	...	...	...	...	...	12.5
28	...	...	...	...	...	13.5	...	...	...	...	...	12.5
29	...	...	...	...	...	11.0	...	...	...	...	...	6.7
30	...	...	...	...	...	3.0	...	...	...	...	...	2.0
31	...	...	...	...	...	3.0	...	...	...	...	...	2.0
32	...	...	...	...	...	3.0	...	...	...	...	...	2.0
33	...	...	...	...	...	12.1	...	...	...	...	...	12.5
34	...	...	...	...	...	10.9	...	...	...	...	...	10.0
35	...	...	...	...	...	11.6	...	...	...	...	...	12.5
36	...	...	...	...	...	14.5	...	...	...	...	...	13.3
37	...	...	...	...	...	21.5	...	...	...	...	...	20.0

toxine-like alkaloids, but only slight traces, if any, of the ergonovine. Incidentally, the more fats and waxes the drug contains, the more difficult it is to separate the alkaloids. The filtrate fraction (ergonovine) after precipitation with sodium bicarbonate still has enough of the ergotoxine-like alkaloids to prevent an oxytocic test. These alkaloids were removed by shaking with carbon tetrachloride (Table II, columns 6 and 12). As previously reported by Hampshire and Page (12), the ergonovine base is insoluble and the ergotoxine base is soluble in carbon tetrachloride. Thus, in the crude drug, ergonovine is more accurately evaluated by this method than the ergotoxine-like alkaloids.

Table III.—Examination of Thirty-six Commercial Fluidextracts of Ergot U. S. P.

	Colorimetric Method					Biological Methods					
	Ergotoxine Ethanesulfonate Equivalent, mg. per 100 cc.				Ergonovine Maleate Equivalent, mg. per 100 Cc.	Broom-Clark Method Ergotoxine Ethanesulfonate Equivalent, mg. per 100 Cc.					Ergonovine Maleate Equivalent, Mgm. per 100 Cc.
F. E. No.	Ether Washings	Total Alkaloids	After Pptn. with $NaHCO_3$	CCl_4 Washings		Original F. E Before	Ether Washings	After Ether Washings	After Pptn. with $NaHCO_3$	CCl_4 Washings	
1	None	111.76	50.88	30.10	11.00	50.0	None	50.0	44.0	5.00	8.0
2	None	95.92	51.80	32.30	10.40	50.0	None	48.5	47.0	3.50	6.0
3	Trace	122.96	52.20	30.00	9.50	50.0	None	55.0	40.0	7.00	4.0
4	Trace	208.60	62.40	36.40	13.70	70.0	None	65.5	50.0	15.00	8.0
5	None	81.60	20.00	16.80	7.70	24.0	None	23.0	20.0	4.25	3.0
6	None	107.20	35.00	25.70	11.00	41.0	None	40.0	30.0	5.00	6.0
7	None	160.00	46.00	36.00	22.80	50.0	None	50.0	40.0	6.35	8.0
8	None	77.60	20.60	23.00	12.20	12.0	None	13.0	11.0	2.55	4.0
9	None	177.12	64.00	41.60	17.20	44.0	None	45.0	40.0	5.65	6.5
10	None	116.52	26.40	19.40	8.00	37.5	None	38.0	35.0	2.95	2.5
11	None	101.50	58.20	29.30	10.00	55.0	None	54.0	50.0	4.00	8.5
12	None	93.90	43.20	17.50	8.60	45.0	None	43.0	38.0	7.00	5.0
13	...	...	49.50	...	6.52	...	...	...	30.0	...	5.0
14	...	...	45.00	...	7.15	...	...	...	27.5	...	5.7
15	...	...	40.00	...	5.10	...	...	...	26.0	...	5.0
16	...	...	51.50	...	5.20	...	...	...	31.0	..	5.0
17	...	...	42.50	...	5.56	...	...	...	26.5	...	3.2
18	...	...	45.50	...	6.00	...	...	...	38.5	...	5.2
19	...	...	51.50	...	5.94	...	...	...	30.5	...	3.0
20	...	...	55.50	...	8.16	...	...	...	30.0	...	5.1
21	...	...	52.50	...	12.10	...	...	...	36.5	...	10.0
22	...	...	60.50	...	13.60	...	...	...	37.5	...	11.0
23	...	...	58.50	...	5.55	...	...	...	41.5	...	5.0
24	...	...	62.20	...	7.95	...	...	...	37.8	...	2.5
25	...	...	62.20	...	6.85	...	...	...	40.0	...	5.0
26	...	...	57.20	...	13.70	...	...	...	37.0	...	11.0
27	...	...	89.50	...	11.60	...	...	...	43.5	...	10.0
28	...	...	89.50	...	18.90	...	...	...	44.5	...	18.2
29	...	...	61.50	...	14.00	...	...	...	42.5	...	8.0
30	...	...	70.00	...	13.00	...	...	...	37.0	...	8.0
31	...	...	80.74	...	17.50	...	...	...	45.0	...	10.0
32	...	...	56.08	...	16.00	...	...	...	40.0	...	8.0
33	...	...	61.44	...	18.00	...	...	...	42.5	...	10.0
34	...	...	36.24	...	13.00	...	...	...	30.0	...	8.0
35	...	...	19.90	...	13.00	...	...	...	15.0	...	9.0
36	...	...	60.48	...	13.00	...	...	...	40.0	...	9.0

Method for the Fluidextract of Ergot, U. S. P.—In the preparation of the fluidextract, 40 per cent alcohol is used. This highly colored dilute alcoholic solution required a slightly different procedure. The method may read as follows:

"Place 12.5 cc. of the fluidextract of ergot U. S. P. in a flask suitable for use in a mechanical shaker. Add 5 cc. of 10 per cent ammonia plus 125 cc. of reagent acetone, shake for one hour, filter, and take 100 cc. of the filtrate, which represents 10 cc. of the fluidextract, and evaporate under a current of air. to dryness (it is necessary to remove all the alcohol). Add 25 cc. of distilled water, make alkaline to a pH of 8, plus 7.5 Gm. of sodium chloride, and shake with three separate 50-cc. portions of ether (10 minutes for each shaking). Under a current of air evaporate the combined ether shakings into approximately 25 cc. of a 2 per cent solution of tartaric acid. To remove coloring matter, shake the acid solution with two 50-cc. portions of ether. Evaporate off the traces of ether by vacuum. Make the acid solution alkaline with sodium bicarbonate to a pH of 8, allow to stand for 10–15 minutes, filter, and wash the container and filter paper with two 10-cc. portions of a 1 per cent solution of sodium bicarbonate. The precipitate on the filter paper represents most of the water-insoluble alkaloids (ergotoxine-like) while the filtrate contains the water-soluble alkaloid (ergonovine). Dissolve the precipitate on the filter paper in 10 cc. of a 2 per cent solution of tartaric acid and assay by the Broom-Clark Method. To remove all traces of the ergotoxine-like alkaloids, shake the filtrate (ergonovine) with two portions of 50 cc. of carbon tetrachloride. After separation, evaporate the last traces of carbon tetrachloride by vacuum (a little heat may be necessary). Saturate

the ergonovine solution with sodium chloride and shake with successive portions (50 cc.) of ether until free from blue color (*p*-dimethyl-aminobenzaldehyde reagent). Finally, evaporate the combined ether fractions into 10 cc. of distilled water, remove last traces of ether by vacuum, pH to 3 with tartaric acid, wash with 50 cc. of ether (to remove last traces of color), again remove ether fumes by vacuum, and assay for ergonovine, biologically and colorimetrically, with pure ergonovine maleate as the standard."

As shown in Table III, in the fluidextract the ergonovine content varied from 2.5–18.2 mgm. per 100 cc. (isolated rabbit's uterus) and 5.1–18.9 mgm. per 100 cc. colorimetrically. On the average, the color test gave a higher value than the bioassay method. Thus, this method is useful in determining the ergonovine potency (both biologically and colorimetrically) in the fluidextract.

For the ergotoxine-like alkaloids in the fluid extract, the method appeared to be more quantitative than for the same alkaloids in the crude drug. Physiologically, the results in Table III (column 7 [original assay] and column 9 [after ether washing to remove color]) checked reasonably close, showing that the ether did not extract (unlike that found in the crude drug) any of the water-insoluble alkaloids. This difference in the drug and fluidextract is probably due to the fact that the fluidextract is more thoroughly defatted than the crude drug. Colorimetrically, the results were not in agreement with the bioassay values. In the fluidextract, therefore, it is possible to evaluate the ergotoxine-like alkaloids biologically but not colorimetrically.

In view of the wide variation in the ergonovine content (2.5–18.2 mgm. per 100 cc.) and the ergotoxine-like alkaloids (12–70 mgm. per 100 cc.) of fluid extracts of ergot U. S. P., and the pharmacological evidence (11) of antagonism between these alkaloids, the U. S. P. Cock's Comb Method is obviously of little value for potency determination. It is also well known among bioassayists that the fluidextract is not a stable preparation. Furthermore, ergonovine now plays an important role in the practice of obstetrics and, together with ergotoxine or ergotamine, is considered the chief alkaloid in ergot. These useful alkaloids are now available in pure form. Thus, the uncertainty of potency and of stability of the fluidextract, as well as the availability of its important alkaloids in pure form, leads one to believe that the fluidextract of ergot U. S. P. has lost much of its usefulness as a true representative of ergot preparations.

SUMMARY

1. An assay process for crude ergot and fluidextract of ergot U. S. P. is described, in which ergonovine is completely separated from the ergotoxine-like alkaloids.

2. By this method thirty-seven lots of crude ergot drug and thirty-six various commercial fluidextracts of ergot U. S. P. were studied.

3. The ergonovine content in these preparations was more accurately evaluated by this method than the ergotoxine-like group.

4. For potency tests on the average, the colorimetric method gave higher values than the biological methods. The isolated rabbit's uterus was used for ergonovine, and the Broom-Clark Method for the ergotoxine-like alkaloids.

5. In the commercial isolation of ergonovine maleate, this method has been helpful in the selection of the crude ergot and in the yield expected during the process of manufacture.

The authors are indebted to Dr. K. K. Chen for his criticism of the manuscript.

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"Brevity in writing is the best insurance for its perusal."—Virchow

The Quantitative Separation of Ergometrine from Other Ergot Alkaloids*

By Donald C. Grove†

Since the discovery of the water-soluble alkaloid, ergometrine (ergonovine), and the recognition of its predominate therapeutic importance, the need of a quantitative method for its determination in ergot and ergot preparations has been imperative. The alkaloids of ergot occur in isomeric pairs, five such pairs having been isolated and identified to date. Only one isomer of each of these pairs is physiologically active, and only one pair, ergometrine-ergometrinine, is water-soluble, the ergometrine representing in this case the physiologically active component. When ergot or an ergot preparation is taken orally, the greater portion of the physiological response is probably due to the water-soluble alkaloid, ergometrine.

The separation of the water-soluble alkaloids from those which are water-insoluble has already been accomplished, Hampshire and Page (1), Trabucchi (2), Allport and Porter (3), Schou and Tonnesen (4), Casparis and Bullet (5) and Schumacher (6). A survey of these methods revealed that none of them was adequate for the quantitative separation of ergometrine from its inactive isomer, ergometrinine. It was evident, therefore, that the present investigation should start with an attempt to separate the two water-soluble alkaloids.

The ergotoxine ethanesulfonate, ergotinine, ergosine, ergosinine, ergometrine and ergometrinine which were used for the experimental work were purchased from Burroughs Wellcome & Co. Ergotamine tartrate was obtained from Sandoz Chemical Works, Inc. A very small sample of ergometrine, prepared in this laboratory from ergot and purified by many recrystallizations from chloroform, was used as a standard for comparing the purchased ergometrine. The optical crystallographic properties of both samples of ergometrine were found to be identical. The ergometrinine had $[\alpha]_D^{20} + 423°$ in chloroform ($c = 0.1$). Smith and Timmis (8) reported a value of $+414°$ for this alkaloid. Because of the small quantities available, all of the above alkaloids were used as purchased without any further attempt at purification.

Allport and Porter (3) made use of an antimony trichloride reagent which precipitated ergometrine, but not ergotoxine. While this work was confirmed, studies with ergometrinine showed that this alkaloid was also precipitated almost quantitatively by this reagent and, therefore, the method would be of no value in the separation of ergometrine from ergometrinine. The procedure of Trabucchi (2) using picric acid was tried and it was found that while ergotoxine was precipitated, ergometrine and ergometrinine remained together in solution. Morrell and Chapman (private communication) have been able to separate the water-insoluble alkaloids from ergometrine by means of a phosphomolybdic-phosphotungstic acid reagent. They had no ergometrinine available to test, but were of the opinion that it probably would remain in solution with the ergometrine. This has been confirmed and, therefore, their reagent would not effect a separation of these two alkaloids.

It was evident from the above that the possibility of finding a reagent that would precipitate one of these isomeric alkaloids, and not the other, was not very promising, so this method of attack was abandoned. The manner in which these two alkaloids distributed themselves between water and various immiscible solvents was studied in the hope of finding a solvent that would effect a separation. The procedure used was as follows:

EXPERIMENTAL

Varying amounts of the alkaloid were dissolved in slightly ammoniacal water (pH = 10), these water solutions were analyzed colorimetrically, using the reagent of Allport and Cocking (7) and a Brice (9) abridged Photoelectric Spectrophotometer (filter for 560 mμ). Aliquots were taken and shaken for 10 minutes with equal volumes of the immiscible

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solvents at room temperature (approx. 25° C.). The water layer was then withdrawn and the amount of alkaloid remaining in the water determined colorimetrically. The results obtained are given in Table I.

Table I.—Distribution of Ergometrine and Ergometrinine between Equal Volumes of Water and Various Immiscible Solvents

Immiscible Solvent	Per Cent Remaining in Water: Ergometrine	Per Cent Remaining in Water: Ergometrinine
Ether (U. S. P.)	88.3	28
Dichlorethylene	43.7	3.4
Chloroform	26.0	3.4
Trichlorethylene	90.3	29
Isopropyl ether	93.4	43
Carbon tetrachloride	100	85
Benzene	81.6	32
Methylene chloride	52.4	23
Ether, 2 vols. / Dichlorethylene, 1 vol.	49	7.6

Of all the solvents tried, ether seemed to offer the most promise in that it causes less emulsification when used with the drug, exists as the top layer in the separator and allows as great a separation of the two alkaloids as any of the other solvents. A consideration of the distribution ratio of ergometrine and ergometrinine between ether and water disclosed that if an ether solution of them was shaken repeatedly with water, all of the ergometrine would be extracted together with considerable ergometrinine. However, if the water extracts were washed successively through a second and a third separator containing ether, most of the ergometrinine would be eliminated. Using the distribution ratio of these alkaloids between water and ether, theoretical calculations were made to determine, using three ether-containing separators, the proper volume ratios of the solvents and the optimum number of water extractions to be made to give the most complete separation. The method finally adopted was as follows:

Twenty-five cc. of a 1% tartaric acid solution of the alkaloids was placed in a separator and made faintly alkaline to litmus with ammonia, 35 cc. of U. S. P. ether was added and the mixture shaken for three minutes by hand. It is essential that this period of shaking be used in making all of the extractions throughout this procedure. The separator was then inverted and the stop-cock cautiously opened to permit the release of excess pressure. The two layers were allowed to separate sharply, and the water layer was drawn off as completely as possible into a second separator containing 35 cc. of ether. The second separator was shaken and the water withdrawn into a third separator containing 35 cc. of ether. After shaking in the third separator, the water was drawn off into a 250-cc. beaker. The whole process described above was repeated with six successive 10-cc. portions of weak ammonia water (prepared by adding one drop of 10% NH_3 W/V to 200 cc. of water, giving a pH of approximately 10), and the water extracts were combined in the 250-cc. beaker. The dissolved ether was removed from the combined water extracts by warming slightly on the steam bath (40–45°) while blowing a current of air over the water, the solution cooled, made to 100 cc. and the alkaloids determined colorimetrically as previously described. The volume ratios of water and ether should be strictly adhered to, as any change in these ratios will affect the percentage distribution of the alkaloids.

One per cent tartaric acid solutions of the various alkaloids were prepared, assayed colorimetrically, and 25-cc. aliquots were run through the above-described procedure. The results obtained when ergometrine and ergometrinine were run separately and when mixed together are shown in Table II, as well as results for a mixture of the five water-insoluble alkaloids and mixtures of all seven of the alkaloids available for this study.

Table II.—Determination of Ergometrine in Various Mixtures

Alkaloid	Amount Taken, Mg.	Recovered in Water Ext. Calc. as Ergometrine: Mg.	Recovered in Water Ext. Calc. as Ergometrine: %
Ergometrine	2.092	2.077	99.3
	1.520	1.494	98.3
Ergometrinine	2.442	0.135	5.5
	2.230	0.107	4.8
Ergometrine + Ergometrinine	0.831 + 0.977	0.881	106
	0.912 + 0.446	0.921	101
Water-insoluble alkaloids[a]	10.943	0	0
Water-insoluble alkaloids +	6.566 + 1.050 + 0.466	1.105	105
	5.926 + 1.009 + 0.504	1.009	100
Ergometrine + ergometrinine	6.914 + 0.504 + 0.504	0.531	105

[a] The water-insoluble alkaloids consisted of approximately equal parts of ergotoxine, ergotinine, ergotamine, ergosine and ergosinine. The figure given is for total alkaloids.

It can be seen from the results in Table II that about 98% to 99% of ergometrine can be recovered and about 5% of the amount of ergometrinine present comes through. This would tend to give slightly high results for ergometrine, the degree depending upon the quantity of ergometrinine present. It was found that in running ergometrinine alone, the last ether wash always contained about 15%

of the amount taken. Therefore, in applying this method to the assay of ergot, the last ether wash can be extracted with 1% tartaric acid and an approximation of the amount of ergometrinine can be obtained. From some preliminary results recently obtained on two different samples of ergot, the ergometrinine present amounted to about half of the ergometrine content. If this is true for all samples of ergot, the error due to ergometrinine would amount to about 2–3%.

None of the five water-insoluble alkaloids tested came through in the water extract. It is regrettable that the remaining three water-insoluble alkaloids, ergotaminine, ergocristine and ergocristinine were not available for testing. It is felt, however, that they will not interfere with the method as they are all water-insoluble, ether-soluble alkaloids.

After the water-insoluble alkaloids (no ergometrine or ergometrinine present) had been run through the ether system, the second and third ether washes were shaken out separately with 1% tartaric acid and the alkaloid determined colorimetrically. It was found that the second ether contained 5.7% of the total amount of alkaloid used at the start of the assay and the third ether contained only approximately 0.2%. This indicates that in use of the Hampshire and Page (1) technique, about 6% of the water-insoluble alkaloids may be present in the water extract. Casparis and Bullet (5) reported as much as 12% of these alkaloids carried over into the water when they used the Hampshire and Page method.

Further work is being done at the present time adapting this separation scheme to the assay of ergot, the results of which will be reported in a future paper. It is hoped that this method will result in an agreement between the chemical and biological assays for ergometrine.

The author is indebted to Mr. Geo. L. Keenan of the Microanalytical Division of The Food and Drug Administration for checking the optical crystallographic properties of the purchased ergometrine.

SUMMARY

A method for the quantitative separation of ergometrine from the water-insoluble ergot alkaloids as well as from its therapeutically inactive isomer, ergometrinine, has been presented.

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A Phytochemical Study of *Aloe vera* Leaf*

By Tom D. Rowe† and Lloyd M. Parks‡

The study reported in this paper was undertaken in connection with an attempt to isolate and characterize the principle contained in *Aloe vera* leaf which is responsible for its activity in promoting the healing of third degree x-ray reactions on white rats. Although dried aloe and its constituents have been the subject of numerous investigations, little work has been done on the fresh aloe leaf. Hence, much of the work reported herein is preliminary in nature. Most of the cursory investigations of the fresh leaf which have appeared from time to time have been of a histological and anatomical nature.

Robiquet, in 1846 (1), reported studies which have since been contradicted. Reports by Unger in 1855 (2), Gasparrini in 1863 (3) and Trecul in 1872 (4) contributed little to our chemical knowledge of the leaf. Prollius in 1884 (5) contributed perhaps the most informative report on several species of aloe, while the latest work of Chopra and Ghosh, in 1938 (6), on fresh *Aloe vera* leaf reported the presence of a trace of volatile oil, non-volatile oil, resin, gum, emodin, an anthraquinone compound, and chrysophanic acid. These constituents, plus aloin, are essentially all that have been found in previous investigations.

* Based on a portion of a thesis submitted to the Faculty of the University of Wisconsin in partial fulfilment of the requirements for the degree of Doctor of Philosophy by Tom D. Rowe.

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Most of the leaf material used in this study was obtained from the Jamaica Gardens of S. B. Penick and Company[1] and was received in shipments throughout the year 1940–1941. Details concerning these shipments appear in another report (7). The investigation was divided into four main parts, namely, preliminary tests on the whole leaf, on the rind, on the pulp, and fractionation of the pulp.

EXPERIMENTAL

Preliminary Tests and Determinations on the Whole Leaf.—The fresh leaves used in this part of the work were obtained in July and August 1937, from the General and Marine Laboratory, Hollywood, Florida, and were in good condition when received. The results are summarized as follows:

1. Moisture Content (8)—96.5%.

2. Selective solvent extraction on a sample dried by distillation with xylene. The percentage of extractive is expressed on the basis of both dried weight and fresh leaf:

	Dried Leaf, %	Fresh Leaf, %
Ether	2.6	0.11
Chloroform	1.1	0.05
Alcohol	8.6	0.38
Water	80.3	3.50

3. Pentosan content (9)—0.5% of fresh weight later work indicated that most, if not all, of the pentosans were contained in the rind.

4. Cellulose content (10)—35.6% (dry weight) or 0.96% on the basis of the fresh weight of the leaf.

5. Qualitative tests showed reducing sugars, both simple and hydrolyzable, present, phenols and tannins absent.

Preliminary Tests and Determinations on the Rind.—All of the rind used in these tests was, with the exceptions noted, obtained from Jamaica during 1941. The results are summarized as follows:

1. Moisture content, determined by drying to constant weight at 105°—for Florida leaves, 82.9%; for Jamaica leaves, 90%. (This difference is probably due to the time of year during which the leaves were cut.)

2. Ash content—for Florida leaves, 3.3%.

3. Enzymes.—Qualitative tests showed the presence of an oxidase and a catalase.

4. Pentosan content—0.55% on basis of fresh weight.

5. Sugars.—The alcohol-soluble portion of the rind was found to contain, before hydrolysis, 16.1%, and after hydrolysis, 22.2% of reducing sugars.

6. Nitrogen content (11).—The dried rind contained 1.39% nitrogen, equivalent to 0.14% on the basis of the fresh rind.

7. Proteins—not present in aqueous or alkaline extracts of the fresh rind.

8. Alkaloids, tannin and aloin—not present in the fresh rind.

9. Sulfur—present in the fresh rind.

10. Phenols—a trace may be present in the fresh rind.

11. Selective solvent extraction of the dried rind.—Rind was dried at 65° for 24 hours, powdered, and extracted to exhaustion with various solvents with the following results:

Solvent	Percentage of Extractive Dry Weight, %	Fresh Weight, %
Petroleum ether	1.86	0.18
Ether	1.71	0.17
Chloroform	0.71	0.007
Alcohol	10.50	1.05
Water	30.20	3.02
2% HCl	24.60	2.46
2% KOH	20.00	2.00
Marc	5.04	0.50
Total	94.62	9.38

The aqueous extract from this portion, upon concentration to a small volume, and the addition of excess alcohol, yielded a precipitate which was dried to an easily pulverized mass and which represented 23.3% of the dried rind. This substance was slightly soluble in water, readily soluble in dilute hydrochloric acid, and insoluble in either sodium or ammonium hydroxide solutions. A saturated aqueous solution of it was slightly more viscous than water and was precipitated by alkalies. The uronic acid content of this substance determined by the method of Dickson, Otterson and Link (12) was found to be 7.34%. This, plus the above information, indicated the material to be a mucilage.

12. Carotene and vitamin A content.—Colorimetric determinations, carried out by the Department of Biochemistry, University of Wisconsin, on the fresh rind, showed the presence of 7.2 micrograms of carotene per gram. No vitamin A was found. This is a normal content of carotene for fresh green leaf materials.

13. Vitamin D content.—This assay was carried out by the laboratory of the Wisconsin Alumni Research Foundation, using the U. S. P. XI procedure (13). Dried rind was extracted with water in order to remove any cathartic principle that might have been present. The rind, thus prepared, and fed at a level of 7 Gm. (equivalent to approximately 70 Gm. of fresh rind) showed no vitamin D activity, indicating the absence of vitamin D in the rind.

14. Aloin content.—Portions of the fresh rind were macerated in alcohol for periods from two to four months, strained, and the residue then extracted with boiling alcohol. Each of these alcoholic extracts was then concentrated to near dryness and the commonly used tests for aloin and related com-

[1] Our thanks are due to Mr. S. B. Penick for his kind coöperation in furnishing the leaves used in this investigation.

pounds applied to them (14). Tests for aloin, emodin and isobarbaloin were positive in both extracts, being only slightly positive, however, in the hot alcoholic extract.

A quantitative aloin determination on the cold alcoholic extract, by the method of Smith and Jordan (15), yielded 4.6% of aloin, equivalent to approximately 0.1% of the fresh rind. This, however, must be considered as only an approximation at best since some of the aloe forming latex had undoubtedly drained out of the leaves upon cutting and during shipping. These results, together with the previous negative test for aloin in the fresh rind, show that aloin is present only after the rind has been allowed to stand. The absence of aloin in the fresh rind is in agreement with the observation of Chopra and Ghosh (6).

Preliminary Tests and Determinations on the Pulp.—The pulp is the jelly-like, colorless material contained within the *Aloe vera* leaf and constitutes approximately two-thirds of the entire weight of the leaf. The fresh leaves used in most of this part of the work were obtained from Florida in August 1939. The results are summarized as follows:

1. pH.—Determined with a Cameron pH meter, the fresh pulp was found to have a pH of 4.7.
2. Moisture content—98.5%.
3. Ash content—0.2%, on basis of fresh weight.
4. Viscosity.—The viscosity of the juice, expressed by hand from the pulp, was approximately equal to a 30% aqueous solution of gum acacia.
5. Enzymes.—Qualitative tests showed the presence of an oxidase and an amylase.
6. Protein and starch—negligible amounts were indicated.
7. Sugars—0.1% of simple reducing sugar was found. No hydrolyzable sugars were found; however, later examination of fractions of the pulp indicated the presence of small amounts of hydrolyzable sugars.
8. Calcium oxalate.—Crystals found imbedded in large amounts in the pulp after macerating in alcohol were identified as calcium oxalate.
9. Pectin and pectase.—Qualitative tests, both chemical and microscopic, by the procedures of Carre and Haynes (16), and Carre and Horne (17), failed to reveal the presence of pectin in either the fresh pulp or any of the fractions obtained from it. Tests for pectase, by the method of Davison and Williman (18), were negative.
10. Carotene and vitamin A.—Colorimetric determinations showed the absence of both of these substances.
11. Vitamin D.—Pulp, dried at 80° for 24 hours, when fed at a level of 5 Gm. (equivalent to approximately 333 Gm. of fresh pulp), showed no vitamin D activity, indicating the absence of this vitamin in the pulp. This is contrary to the statement of MacKee (19) that the healing property of *Aloe vera* pulp in the treatment of third degree X-ray reactions is thought to be due to vitamin D.

Fractionation of the Pulp.—All of the material used in this part of the work was obtained from Jamaica in three shipments during the months of July, August and September 1940. The pulp was removed from the leaves of each shipment by cutting and scraping with a spatula. In the case of the first two shipments it was pickled in alcohol and set aside for future use. The pulp from the third shipment was examined while fresh.

Fresh Pulp.—The pulp, freed from rind, was run through a meat chopper, expressed through gauze by hand, and the expressed juice concentrated by distilling under reduced pressure at a temperature of 65–70°. The aqueous distillate, which was not examined further, possessed a pleasant aromatic odor, indicating the presence of a volatile substance. To the concentrated juice remaining in the still was added five and one-half times its volume of alcohol, resulting in the precipitation of a fine, sponge-like substance, which settled out upon standing and was filtered, the hydro-alcoholic filtrate being concentrated to dryness. From the original pulp there were thus obtained three fractions:

1. The expressed pulp, preserved by placing in alcohol.
2. The alcohol-insoluble portion of expressed juice.
3. The hydro-alcoholic filtrate, not examined further at this point.

The gum-like, alcohol insoluble portion of the expressed juice was washed with alcohol and, when dried at 65° for 24 hours, was equivalent in weight to 0.1% of the fresh whole pulp. The ash content of this substance was found to be 9.5%, and the uronic acid content, 4.7%. This fact, together with its slight solubility in water, insolubility in alcohol and other physical properties, indicated the substance to be a mucilage (20, 21).

Pulp Macerated in Alcohol.—As previously mentioned, the pulp from the first two shipments of leaves was removed and allowed to macerate in alcohol for about three months. The mixture was then strained through gauze and the solid residue expressed by hand, separating the original pulp into two fractions:

1. The hydro-alcoholic solution of expressed juice.
2. The alcohol-extracted and expressed pulp.

1. To the hydro-alcoholic solution of expressed juice was added sufficient alcohol to cause complete precipitation. The precipitate formed in two layers, one on top and one on the bottom. Since there was the possibility of their being different in nature they were collected separately and, for the most part, examined separately. They were first air-dried and then dried at 65° for 24 hours. The hydro-alcoholic filtrate remaining after the above precipitation was evaporated to near-dryness under reduced pressure, resulting in a dark brown semi-solid mass.

Results of qualitative tests on these three portions are summarized as follows:

	Alcohol-Insoluble Portion		Concentrated Hydro-Alcoholic Filtrate
	Top Portion	Bottom Portion	
1. Nitrogen (11)	+	+	−
2. Protein (22, 23)	−	−	−
3. Halogen (11)	+	−	−
4. Molisch test	+	+	+
5. Phenols	−	−	−
6. Alkaloids[a]	−	−	−
7. Calcium	+	+	+
8. Reducing sugars			
Before hydrolysis	−	−	+
After hydrolysis	+	+	+
9. Aloin and emodin	−	−	−

[a] Tannic acid, T.S., and Phosphotungstic acid, T.S., produced marked coagulations but all of the other alkaloidal reagents were negative.

Results of quantitative determinations on these three portions are summarized as follows:

	Alcohol-Insoluble Portion, %		Concentrated Hydro-Alcoholic Filtrate, %
	Top Portion	Bottom Portion	
1. Nitrogen	0.55	0.70	Not determined
2. Reducing sugars (glucose)			
Before hydrolysis	0[a]		43.95
After hydrolysis	33.96[a]		43.95
3. Pentosans	0.95	0.98	2.15
4. Crude fiber (24)	0.30[a]		Not determined

[a] The top and bottom portions were combined for these determinations since the previous tests had shown them to be the same substance.

Qualitative tests on the dark brown, semi-solid mass resulting from concentration of the *hydro-alcoholic filtrate* remaining after the above precipitation showed the absence of pentoses and the presence of a ketose sugar. When treated by the benzimidazole condensation procedure of Moore and Link (25) for the identification of aldoses the results were negative, showing the absence of an aldohexose. The formation of a phenylosazone, melting at 208°, plus the positive tests for a ketose obtained previously and the elimination of an aldohexose by the benzimidazole procedure, confirmed the presence of fructose in this residue. This fact, together with the absence of a pentose, indicated that the results obtained previously in pentosan determinations were probably due to a partial degradation of the hexose during the acid distillation of the pentosan procedure.

2. *The alcohol-extracted and expressed pulp*, after being dried and powdered, was extracted with selective solvents with the following results, the percentage of extractive being expressed on the basis of both the dry expressed pulp and the fresh pulp.

	Dry Pulp, %	Fresh Pulp, %
Ether	0.48	0.002
Alcohol	8.80	0.03
Water	26.60	0.09

The ether and alcohol extractives were too small in amount to permit further examination. The aqueous extract, upon evaporation to a small volume, and addition of excess alcohol, yielded a precipitate which, upon drying and examination, was identified as the same mucilage obtained previously from the hydro-alcoholic solution of the expressed juice. The filtrate, when tested for reducing sugars, showed the presence of a reducing substance which was not a sugar. It reduced potassium permanganate solution and appeared to contain a carbonyl group, although attempts to prepare an oxime, a semicarbazone or a phenylhydrazone were unsuccessful. Although it was impossible to isolate it in crystalline state, evidence pointed to the fact that it was probably the soluble salt of a uronic acid.

SUMMARY AND CONCLUSIONS

1. Results are given for preliminary tests on the whole leaf, rind and pulp of *Aloe vera*. The alcohol-insoluble portion of the leaf was found, from its uronic acid content, to be a mucilage. The presence of simple reducing sugars and hydrolyzable sugars was shown. Fructose was identified as the simple reducing sugar.

2. An oxidase, a catalase and an amylase were found. Pectin, alkaloids, phenols, tannins, vitamin A and vitamin D were shown to be absent. Aloin was not found in either the fresh pulp or fresh rind, but was found in small amounts in the rind which had macerated for a long time in alcohol.

3. The absence of tannin, pectin, vitamin A, vitamin D and the small amount of nitrogenous substances found in the leaf or any concentrated fraction of it, proved that the healing property of *Aloe vera* leaf is not due to any of these principles or to urea.

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Book Review

Laboratory Instruction in Biochemistry, by ISRAEL S. KLEINER and LOUIS B. DOTTI. 188 pages. $7^3/_4$ x $10^1/_2$ (loose leaf). 1940. St. Louis: C. V. Mosby Co. $1.50.

This is a collection of laboratory experiments in elementary biochemistry on individual punched sheets which can be included in a laboratory notebook. The collection is intended for medical students and includes simple directions for elementary experiments in the biochemistry of carbohydrates, fats, proteins, milk, blood, urine, metabolism, food, etc. While intended primarily for medical students, it should also be of interest to pharmacy teachers and students.—A. G. D.

Further Observations on the Use of *Aloe vera* Leaf in the Treatment of Third Degree X-Ray Reactions*

By Tom D. Rowe†, B. K. Lovell‡ and Lloyd M. Parks**

In 1940 one of us (T. D. R.) published a preliminary report on the use of fresh *Aloe vera* jell in the treatment of third degree x-ray reactions on white rats (1). Attention was called at that time to the modern use of this leaf in the treatment of such reactions in humans. It should also be pointed out that various species of *Aloe* have been employed for centuries to promote the healing of wounds and fire burns. Thus, Turner in 1568 (2) cited the use of "the herbe Aloe is to hele wounds," and Coxe in 1818 (3) referred to the use of powdered aloe "to check heamorrhagies in recent wounds." Various other references have from time to time mentioned aloe, either fresh or dry, as a healing agent. The present report deals with further observations on the use of the fresh jell, or pulp, of the leaf, as well as other portions of the leaf, in the treatment of experimentally produced third degree X-ray reactions on the skin of white rats.

The procedure followed in producing the X-ray reactions and the method of treatment in the present report are essentially the same as those described in the first report, with the following differences: rats were given a single dose of 4000 r instead of in divided doses since it was found that a single dose at this level was not toxic; ether was used for anesthesia in place of pentobarbital; control areas received no treatment of any

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kind; the duration of treatment was from two to four weeks.

The leaves used in the present report were obtained from the Jamaica gardens of S. B. Penick and Company.[1] They were sent in separate shipments of 50 to 155 each at intervals during the year 1940–1941 and were, with the exception of the last two shipments, in excellent condition when received. The following table summarizes the information concerning the various shipments.

Table I. *Aloe vera* Leaves Used

Shipment No.	Number of Leaves	Date Received	Condition on Arrival	Used in Treating Rat Group No.
2	55	8/9/40	Good	6
3	100	9/19/40	Good	7 and 8
4	105	11/8/40	Good	9
5	80	3/14/41	Good	10 and 11
6	155	3/31/41	Poor[a]	12, 13, 14 and 15
7	90	4/18/41	Poor[a]	12, 13, 14, 15 and 16

[a] Many of the leaves of these shipments were completely decomposed and had to be discarded. The boxes in which they were shipped were stained a deep pink, indicating that much of the juice or latex from the rind had drained out enroute.

EXPERIMENTAL

Treatment with Fresh Pulp (Jell).—Three groups of rats were treated with this material. Group 6 was treated twice daily for four weeks while Groups 7 and 8 were treated twice daily for three weeks. The shorter period of treatment was resorted to because it was found that spontaneous healing affected the results after three weeks. The results are summarized in Table II.

Table II. Treatment with the Fresh Pulp

Rat Group No.	Improvement with Treatment Definite	Improvement with Treatment Slight	No Difference	Improvement of Control Areas Definite	Improvement of Control Areas Slight
No. 6 14 rats	8	3	3	0	0
No. 7 16 rats	8	2	6	0	0
No. 8 14 rats	5	2	4	1	2
Totals	21	7	13	1	2

By combining the results in the first two columns it may be seen that 28 areas, or 63.8% of those treated, showed an increased rate of healing. This is 9⅓ times as many as showed improvement in the control areas. A significant observation, made during this part of the study, was that those treated areas which showed an increased rate of healing usually did so within two weeks after treatment was started. If no beneficial results had been obtained within this period, continuation of the treatment seldom proved of benefit.

[1] Our thanks are due Mr. S. B. Penick for his kind coöperation in furnishing the leaves used in this study.

By combining the results obtained in the present study with those from the previous report (1) a complete summary of the treatment of a total of 72 rats with the fresh pulp may be shown as in Table III:

Table III. Treatment of 72 Rats with Fresh Pulp

No. of Rats Treated	Improvement with Treatment Definite	Improvement with Treatment Slight	No Difference	Improvement of Control Areas Definite	Improvement of Control Areas Slight
72	31	11	21	2	7

From this it may be seen that a total of 42 areas, or 58.3% of those treated, showed an increased rate of healing. This is 4⅔ times as many as showed improvement in the control areas, and is a definite indication that a healing agent is present in the fresh pulp of *Aloe vera* leaf.

Treatment with the Partially Decomposed Pulp.—Some of the leaves from Shipment 4, after standing at room temperature for one month, were partially decomposed, while parts of them were completely spoiled. The latter areas were cut out and the remainder of the partially decomposed leaves stored at 5° C. After two months the pulp from these leaves had acquired a pink color and a characteristic fermented odor, and had lost most of its viscid, gummy consistency. Treatment of a group of 8 rats (Group 9) with this partially decomposed pulp resulted in 7 areas, or 87.5% of those treated, showing an increased rate of healing, while only one area, or 12.5%, showed no benefit. Moreover, all of the treated areas which responded did so within 12 days after treatment was started. Thus 3 areas were healed in 6 days, 1 area in 9 days, 2 areas in 11 days, and 1 area in 12 days. These results were better than those obtained with the fresh pulp and pointed to the conclusion, contrary to the statement of MacKee (4), that the pulp does not have to be fresh in order to be effective.

Treatment with the Fresh Rind.—The promising results obtained with both the fresh pulp and the partially decomposed pulp led us to suspect that the healing agent might be present originally in the rind and upon standing pass from the rind into the pulp. Accordingly, a group of 8 rats (Group 10) was treated with fresh rind prepared from Shipment 5 to study its effectiveness.

The rind was prepared by removing the pulp from the inside of the leaves by scraping, running the pulp-free rind through a meat grinder, and

thoroughly triturating the minced rind in a mortar, adding one ounce of distilled water to each four ounces of minced rind. This mixture was then applied in the form of a poultice to the areas which had been X-rayed in the usual manner.

All of the 8 areas, or 100%, thus treated were completely healed in 6 days, 5 areas having healed in 5 days. In order to obtain further data on the fresh rind, 4 of the control, or posterior, areas in this group were treated with the mixture, using the 4 remaining posterior areas as controls. Of these 4 posterior areas on which treatment was started 5 days later than usual, two areas were completely healed after 5 days' treatment and the other two were completely healed after 7 days of treatment.

Thus all of the 12 areas (8 anterior and 4 posterior), or 100%, treated with the fresh rind showed a definite increase in the rate of healing. These results, the best obtained with any portion of the leaf, seemed to indicate that the healing agent is present in higher concentration or in a more effective form in the fresh rind of the leaf.

Treatment with Aqueous Extracts of the Dried Rind from Shipment 6.—The positive results obtained with the fresh rind from Shipment 5 pointed to the fact that the healing agent was concentrated in the rind. If the fresh rind could be dried without loss of its effectiveness, then the dried rind could be made available for treatment of X-ray reactions in the form of an aqueous extract or poultice. Hence it seemed desirable to study the effect of drying on the rind and to see if the healing agent were water-soluble. Accordingly, the supply of leaves from Shipment 5 having been exhausted, two aqueous extracts were made from leaves of Shipment 6. The first was made by drying finely chopped rind in a vacuum desiccator for 72 hours, grinding the product to a fine powder, macerating the powder for 10 days with sufficient water to make a 10% mixture, and filtering. The second was made by drying finely chopped rind in an oven at 70° for 24 hours, powdering, macerating the powder with 9 times its weight of water at 70° for 3 hours, and filtering.

The filtrates in each case were used in the treatment of two groups of 8 rats each (Group 12) by saturating cotton packs and placing them on the irradiated areas. Neither of the extracts thus obtained produced an increase in the rate of healing. This would indicate that the healing agent, if present, was either insoluble in water or unstable to drying and extraction. However, these indications could not be conclusive until the fresh rind, from which the extracts were prepared, was shown to be either effective or ineffective as a healing agent.

Treatment with the Fresh Rind of Shipments 6 and 7.—In view of the disappointing results obtained in the preceding experiment with aqueous extracts of the dried rind from these shipments, it was considered desirable to test the effectiveness of the fresh rind itself from these shipments. The fresh rind was prepared, as previously described, by removing the pulp, mincing the rind, and making a poultice by triturating it with distilled water. Two separate groups of 8 rats each (Groups 14 and 15) were treated with this material. Of these only 3 areas, or 18.7% of those treated, showed an increased rate of healing, the other treated areas showing no change.

These results explain the failure to obtain positive results with the aqueous extracts of the dried rind prepared from Shipments 6 and 7 in the preceding experiment and also seem to contradict directly the excellent results obtained with the fresh rind of Shipment 5. However, two possible explanations are offered for these negative results. The first is in the poor condition of the leaves of Shipments 6 and 7 when received Most of the juice, or latex, had drained from the leaves during shipment and it is probable that the healing agent was lost in this way. The second, suggested by a producer of *Aloe vera* leaves (5), is that the healing agent may not be present in the leaves at all times but is found there only during certain seasons of the year.

Treatment with the Partially Decomposed Pulp from Shipment 7.—In order to determine whether the healing agent might have passed from the rind of these leaves into the pulp, a group of 8 rats (Group 16) was treated with the partially decomposed pulp from this shipment. Again, as was the case with the fresh rind and aqueous extracts of the dried rind from this shipment, disappointing results were obtained, only 2 areas, or 25% of those treated, showing an increase in the rate of healing.

Clinical Use of Fresh Rind from Shipments 6 and 7.—After the excellent results obtained from the experimental use of the fresh rind from Shipment 5, it was thought desirable to try the fresh rind clinically on a patient who had been suffering from an X-ray reaction of 7 months' duration and which had failed to respond to any treatment. Unfortunately, however, all of the leaves from Shipment 5 had been exhausted and only leaves from Shipments 6 and 7 were available. Twice daily treatments for one month with a poultice made by mincing the fresh rind with water failed to produce any response in the area involved. This negative result was in line with the same disappointing results obtained in the rat treatments, which were being run simultaneously with the clinical case, with leaf materials from these two shipments.

Treatment with Ointment of Dried Aloe.—In 1937 and 1939 Crewe (6, 7) reported on the beneficial results obtained by treating pruitus vulvae, ivy poisoning, and various types of fire burns with ointments of powdered aloe. These reports led us to try the effectiveness of an ointment of aloe on third degree X-ray reactions. An ointment of Curaçao aloe was made according to Crewe's directions consisting of powdered aloe, 2 drams, mineral oil, 2 drams, white petrolatum, 1 ounce. In a group of 7 rats receiving twice daily treatments for three weeks of this ointment none of the areas showed an increased rate of healing. These results showed that the particular sample of aloe used contained no healing agent for this type of reaction.

Treatment with Scarlet Red Ointment.—A 5% scarlet red ointment is sometimes used in the treatment of X-ray reactions on humans. In order to learn its effectiveness on the test animals, a 5% ointment of Biebrich's medicinal scarlet red in a petrolatum base was used in treating a group of 7 rats twice daily for 3 weeks. Of these none of the treated areas showed an increased rate of healing and in some the time of spontaneous healing appeared to have been prolonged.

Treatment with Urea Ointment.—Various reports (8, 9, 10, 11) in recent years have dealt with the effectiveness of urea in the healing of various types of wounds. In order to study its effectiveness in the treatment of third degree X-ray reactions, an ointment containing urea, 15 Gm., water, 10 cc., aquaphor, *q. s. ad* 100 Gm., was made and applied twice daily to a group of 8 rats. Of these only one area, or 12½% of those treated, showed an increased rate of healing. These results indicate that urea is ineffective in such treatment and, furthermore, that urea is not the healing agent in the *Aloe vera* leaf.

SUMMARY AND DISCUSSION[2]

1. Further observations are reported on the use of *Aloe vera* leaf in the treatment of third degree X-ray reactions on white rats. Sufficient data have been obtained to show that treatment with the pulp of the leaf definitely increases the rate of healing of such experimentally produced reactions. Furthermore, contrary to previous views, our results indicate that the pulp does not have to be fresh in order to be effective as a healing agent. It should be noted that all of the reactions produced in this study are of the acute type and not of the chronic type which sometimes appear on humans many months after the original irradiation.

2. One source of error was noted during the study: The anterior areas of the rats were more resistant to irradiation than the posterior areas. The anterior areas were sometimes smaller than the posterior at the beginning of treatment; consequently, the former occasionally healed normally at a faster rate than the latter. This fact makes the observed results for increased rate of healing somewhat high for all groups of rats treated, but in the later parts of the study the error was overcome to a certain extent by treating alternate anterior and posterior areas.

3. Contradictory results were obtained with the fresh rind of the leaf, the rind from one shipment of leaves being more effective than the pulp in promoting healing, while the rind from two other shipments of leaves gave negative results. The different season of the year in which the latter shipments were collected, or, perhaps more likely, the poor condition of these leaves when received, is offered as a possible explanation for these negative results. It is believed, from our observations, that the healing agent of the leaf is concentrated in the rind. Further work is under way to clear up this question.

4. Results obtained with aloe ointment, scarlet red ointment and urea ointment show that none of these are effective in promoting healing of acute third degree X-ray reactions in the skin of white rats.

[2] Our thanks are due Dr. E. A. Pohle, Professor of Radiology, Univ. of Wis., for his kind coöperation in this study.

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CORRECTION

July, 1941, Issue, page 181.

Change heading of list of compounds in second column from "Substituted Acetylureas" to "Substituted Acetamides."

Fluorophotometric Determinations of Riboflavin*

By Wm. S. Jones and W. G. Christiansen

A number of methods, microbiological as well as chemical, have been employed to assay products and materials for riboflavin. For several years we have been working with the method of Hodson and Norris (1). It involves sulfuric acid extraction of the riboflavin; removal of foreign pigments by mild reduction, followed by mild oxidation with air; and photoelectric measurement of the fluorescent green light, produced when a solution of riboflavin is excited by a blue light. Our experience with this method, the accumulation of information regarding the factor of color in the solutions and data on substances highly fortified with riboflavin have enabled us to modify and simplify the procedure; phosphoric acid has been adopted instead of sulfuric acid as the extracting agent and the reduction-oxidation steps have been omitted. The phosphoric acid acts not only as the extracting agent but also forms part of the buffer system later on in the process; this was helpful because riboflavin gives maximum fluorescence at pH 7.0.

METHOD

A. Instrument.—We have used the Pfaltz and Bauer Fluorophotometer, Model A[1] with a G. E. Mercury Lamp, Type H3, 85 Watts as the source of light. Filter glass (Jena) combination GG-15 and BG-12, giving a peak at 4500 A., is placed between the solution in the 4-cm. glass cuvette and the source of light. An orange filter glass 315, Corning, is placed between the cuvette and photocell. The iris diaphragm, controlling the amount of light admitted to the solution in the cuvette, is opened about 3 cm.

The intensity of the activating light is regulated to give a definite galvanometer reading. This may be done in two ways: first, by using a standard solution of quinine activated by ultraviolet light at 3650 Å. (this requires switching of filter glasses during testing period), or, second, by direct galvanometric setting for the transmitted light picked up by the rear photocell. Once the resistance has been set for a definite fluorometric reading, the intensity of light is held constant by opening or closing iris diaphragm, with a voltage stabilizer in the line. This change in intensity of light is very small.

B. Reagents.—Standard Riboflavin Solution A: Fifty micrograms of anhydrous riboflavin are dissolved in 500 cc. of 25% aqueous alcohol containing 2.0 cc. of 85% phosphoric acid. The solution is kept in an amber bottle in refrigerator. Each cc. contains 100 micrograms riboflavin.

Standard Riboflavin Solution: Prepared daily by diluting 5 cc. of standard solution and 2.0 cc. of 85% phosphoric acid to 500 cc. with distilled water.

N/10 Sodium hydroxide.

Sodium hydrosulfite solution: One Gm. of sodium hydrosulfite and 1 Gm. of sodium bicarbonate are dissolved in 20 cc. of ice-cold distilled water and kept in an ice bath.

Table I.—Results Obtained with the Hodson-Norris Method

	Bioassay, or Expected Value		Found by Hodson-Norris Method	
Riboflavin concentrate, powder	3.0	mg./Gm.	3.193	mg./Gm.
	4.0	mg./Gm.	4.07	mg./Gm.
Liver concentrate	170	μg./Gm.	90	μg./Gm.
Yeast powder, type A	75	μg./Gm.	64	μg./Gm.
	85	μg./Gm.	61.6	μg./Gm.
	85	μg./Gm.	50.7	μg./Gm.
	75	μg./Gm.	59.2	μg./Gm.
	85	μg./Gm.	54.7	μg./Gm.
Yeast powder, type B	43.5	μg./Gm.	36.6	μg./Gm.
Power containing B complex, type A	350–425	μg./Gm.	117.0	μg./Gm.
Powder containing B complex, type B	65	μg./Gm.	52.0	μg./Gm.
	70	μg./Gm.	51.0	μg./Gm.
	75	μg./Gm.	83.0	μg./Gm.
Powder containing B complex, type C	200	μg./Gm.	193	μg./Gm.
	200	μg./Gm.	226	μg./Gm.
	250	μg./Gm.	216	μg./Gm.
	250	μg./Gm.	198	μg./Gm.
Yeast concentrate, liquid, type A	165–180	μg./cc.	71.6	μg./cc.
Yeast concentrate, liquid, type B	58	μg./cc.	50.0	μg./cc.
	78	μg./cc.	62.0	μg./cc.
Yeast concentrate, liquid, type C	250	μg./cc.	124	μg./cc.
Multiple vitamin capsule, type A	114	μg./cap.	112	μg./cap.
Multiple vitamin capsule, type B	1250	μg./cap.	1200	μg./cap.

C. Procedure.—To the sample (1 to 2 Gm. of powders, 1 to 2 cc. of liquids or 2 capsules) in a 250-cc. Erlenmeyer flask are added 100 cc. of distilled water and 0.4 cc. of 85% phosphoric acid solution. The flask is shielded from light by means of close-fitting blackened tin. The mixture is heated on the steam bath for one hour, cooled and filtered by means of paper into a volumetric flask. Any insoluble matter on the filter is washed with distilled water. The filtrate is made up to such a volume that each cc. will contain about 1 μg. of riboflavin; 0.4 cc. of 85% phosphoric acid is added for each 100 cc. additional volume over and above the original 100 cc. of water added. The measurement

* From the Development Division of the Chemical and Pharmaceutical Laboratories of E. R. Squibb and Son, Brooklyn, N. Y. Presented before the Scientific Section of the A. PH. A., Detroit meeting, 1941.

[1] A newer type is now available.

of fluorescence is carried out as follows having the instrument regulated as described above:

(*a*) To 2 cc. of the solution on test, in a 25-cc. amber cylinder, are added 2 cc. of the $N/10$ sodium hydroxide and distilled water to make 25 cc.; 12.5 cc. of this solution are placed in the 4-cm. cuvette and a galvanometer reading is recorded.

(*b*) Two cc. of the Standard Riboflavin Solution B are treated as in (*a*).

(*c*) To a second 2-cc. portion of the solution on test in a 25-cc. amber cylinder are added 2 cc. of Standard Riboflavin Solution B, 4 cc. $N/10$ sodium hydroxide and sufficient distilled water to make 25 cc. Measurement carried out as under (*a*).

(*d*) Repeat (*c*) except that 2 cc. of the hydrosulfite solution are added prior to making up to 25 cc. with distilled water. This constitutes the blank.

(*e*) To 2 cc. of the Standard Riboflavin Solution B are added 2 cc. of $N/10$ sodium hydroxide solution, 2 cc. of the hydrosulfite solution and sufficient distilled water to make 25 cc. Then 12.5 cc. of this solution are placed in the cuvette and fluorescent reading on the galvanometer is recorded. This constitutes a blank, which varies from 1.5–3.5. The difference between (*b*) and (*e*) equals the reading for 1 μg. of riboflavin.

$$\mu\text{g. riboflavin} = \frac{a-d}{b-e} \times \frac{b}{c-a} \times \text{dilution} \times \frac{1}{\text{am't of sample}}$$

Results obtained by meticulously following the Hodson and Norris method are found in Table I. The agreement with the biological or expected values is excellent with some samples and fair with others but in still others the difference is extremely large. Results obtained by the phosphoric acid method are found in Table II and in most cases the stated riboflavin content and found values are in very good agreement.

Five samples of yeast powder, type A, and one of powder containing B complex, type C, were assayed by both the Hodson and Norris and the simplified phosphoric acid procedures. The results, listed in Table III, obtained by the two methods are in fair agreement although somewhat lower than the biological assay figures.

Table II.—Results Obtained with the Phosphoric Acid Extraction Method

	Bioassay, or Expected Value	Found by Phosphoric Acid Extraction Method
Riboflavin concentrate, powder	2.0 mg./Gm.	1.985 mg./Gm.
Liver concentrate	170 μg./Gm.	150 μg./Gm.
Yeast powder, type A	75 μg./Gm.	57 μg./Gm.
	85 μg./Gm.	67 μg./Gm.
	85 μg./Gm.	64 μg./Gm.
	75 μg./Gm.	57 μg./Gm.
	85 μg./Gm.	62 μg./Gm.
Powder containing B complex, type C	250 μg./Gm.	202 μg./Gm.
	260 μg./Gm.	194 μg./Gm.
	230 μg./Gm.	205 μg./Gm.
	270 μg./Gm.	180 μg./Gm.
Powder B complex in capsules	575 μg./cap.	530 μg./cap.
	575 μg./cap.	555 μg./cap.
	575 μg./cap.	530 μg./cap.
	575 μg./cap.	550 μg./cap.
Paste in capsules, type I	5.0 mg./cap.	5.0 mg./cap.
	1.0 mg./cap.	0.94 mg./cap.
	1.0 mg./cap.	0.92 mg./cap.
Paste in capsules, type II	5.0 mg./cap.	5.0 mg./cap.
	5.0 mg./cap.	4.9 mg./cap.
	1.0 mg./cap.	1.02 mg./cap.
	1.0 mg./cap.	1.01 mg./cap.
Paste in capsules, type III	1.0 mg./cap.	0.95 mg./cap.
	1.0 mg./cap.	0.98 mg./cap.
	1.0 mg./cap.	1.00 mg./cap.
	1.0 mg./cap.	1.03 mg./cap.
Paste in capsules, type IV	1.25 mg./cap.	1.15 mg./cap.
	1.25 mg./cap.	1.16 mg./cap.
Paste in capsules, type V	2.5 mg./cap.	2.4 mg./cap.
Paste in capsules, type VI	1.0 mg./cap.	1.05 mg./cap.
Paste in capsules, type VII	125 μg./cap.	124 μg./cap.
	125 μg./cap.	122 μg./cap.
	125 μg./cap.	130 μg./cap.
Paste in capsules, type VIII	25 μg./cap.	28 μg./cap.
	25 μg./cap.	26 μg./cap.
Paste in capsules, type IX	125 μg./cap.	130 μg./cap.
	125 μg./cap.	130 μg./cap.
Paste in capsules, type X	312 μg./cap.	330 μg./cap.
Paste in capsules, type XI	125 μg./cap.	115 μg./cap.

The items listed as Paste in capsules are in all instances multivitamin mixtures with suitable diluents and vehicles in soft elastic capsules.

Table III.—Comparison of Hodson and Norris Method and Phosphoric Acid Method

	Bioassay, or Expected Value	Found	
		Hodson-Norris	Phosphoric Acid
Yeast powder, type A	75 μg./Gm.	64 μg./Gm.	57 μg./Gm.
	85 μg./Gm.	62 μg./Gm.	67 μg./Gm.
	85 μg./Gm.	51 μg./Gm.	64 μg./Gm.
	75 μg./Gm.	59 μg./Gm.	57 μg./Gm.
	85 μg./Gm.	55 μg./Gm.	62 μg./Gm.
Powder containing B complex, type C	250 μg./Gm.	216 μg./Gm.	202 μg./Gm.

Table IV.—Samples Prepared by Enzymatic Digestion

	Bioassay, or Expected Value	Found
Liver concentrate	170 μg./Gm.	100 μg./Gm.
Powder containing B complex, type A	350–425 μg./Gm.	118 μg./Gm.
Yeast concentrate, liquid, type A	165–180 μg./Gm.	121 μg./Gm.
Yeast concentrate, liquid, type C	250 μg./Gm.	144 μg./Gm.

In connection with the fluorophotometric assaying of some materials for B_1 by a procedure in which diastatic digestion is employed, portions of the solutions were tested for riboflavin by the Hodson and Norris method. The data are given in Table IV; they compare fairly well with those obtained by direct application of the Hodson-Norris method (Table I) excepting in one case.

Some 25 lots of synthetic riboflavin have been tested. The solutions were made up in the same manner as the standard solution of riboflavin and compared with the standard riboflavin.

4.75%	of the samples assayed	950,000 μg./Gm.
4.75%	of the samples assayed	960,000 μg./Gm.
9.5%	of the samples assayed	970,000 μg./Gm.
28.6%	of the samples assayed	980,000 μg./Gm.
19.5%	of the samples assayed	990,000 μg./Gm.
33.0%	of the samples assayed	1,000,000–1,020,000 ug./Gm.

Of this series 81% showed 980,000 μg. or better per Gm. of material.

SUMMARY

1. The method of Hodson and Norris for the fluorophotometric determination of riboflavin and a modification of it have been applied to both simple preparations and complex mixtures, natural and artificial.

2. Fair agreement between biological and fluorophotometric determination of riboflavin exists.

The authors appreciate the help of Mr. Frank Howland, Development and Control Laboratory, in carrying out the determinations of riboflavin.

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Book Review

Traffic in Opium and Other Dangerous Drugs for the Year Ended December 31, 1940. Bureau of Narcotics, U. S. Treasury Department. 105 pages. 1941. Washington, D. C.: Government Printing Office. Price, paper, 25 cents.

This publication is the annual report of the Commissioner of Narcotics of the United States. It contains detailed information regarding raw materials, manufactured drugs, new legislation, administration of the Uniform Narcotic Drug Act, control of international trade and coöperative efforts of the nations of the world to control illicit traffic. The number of known medical drug addicts is estimated not to exceed 1 in 3000 population, which represents a reduction of at least 66 per cent in the past two decades. The report should make interesting reading for pharmacists and others interested in the problems of drug addiction.—A. G. D.

Photoelectric Determination of Nicotinic Acid*

*By Wm. S. Jones**

Nicotinic acid, nicotinamide or compounds containing the nicotinic acid nucleus play an important nutritional role as the black tongue and pellagra-preventing vitamin. Recognition of this makes it highly desirable that a rapid method be available for the assaying of materials and products containing this factor.

Many investigators have studied the cyanogen-bromide method of determining nicotinic acid. Harris and Raymond (1) and Kodicek (2) considered *p*-amino acetophenone a more satisfactory amine for this purpose. Kodicek studied further the various factors affecting the reactions and developed a satisfactory method for the extraction and determination of nicotinic acid. The sodium hydroxide hydrolysis to which the sample is subjected gives rise to highly colored substances that interfere badly with the subsequent measurement. Melnick and Field (3) overcame this difficulty by preferential adsorption of the colored substances on Darco G-60 also known as Darco, Coleman and Bell. In our hands this procedure for removal of color gave rise to a loss of nicotinic acid and we did not pursue it further. Other investigators, Arnold, Dehreffley and Lipsuirs (4), have followed the practice of extracting the colored cyanogen-amine compound of nicotinic acid with ethyl acetate and measuring the color photoelectrically. However, even under these conditions the blanks show more color than is desirable. Furthermore, the extra operations incident to the ethyl acetate extraction are time consuming. Moreover, we find that the intensity of the color in the ethyl acetate is subdued and is less, *i. e.*, $^1/_2$ to $^1/_3$, than that in aqueous alcoholic solutions and this is a disadvantage.

Giri and Naganna (5) describe a method in which the foodstuff is hydrolyzed with

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sodium hydroxide, the nicotinic acid adsorbed on medicinal charcoal, then eluted with an alcoholic-sodium hydroxide solution and estimated by the cyanogen-*p*-amino-acetophenone reaction.

By means of nitrous acid, nicotinamide can be converted to the acid. Inasmuch as the factor needs to be in the form of nicotinic acid for measurement by this cyanogen-bromide method, this reaction serves as a means of making it possible to assay materials in which the amide is present.

There is also the possibility that enzyme hydrolysis might be used in place of the alkali or acid hydrolysis and the nitrous acid conversion of the amide as a means of preparing the material for assay. Thus, Thakur and Norris (6) have shown that an amidase, isolated from aspergillus flavus, splits off ammonia quantitatively, from asparagine at a pH 6.3–8.5. Also, in studying the "amide-splitting" ability of yeasts, Gorr, Gunther and Toagner (7) have shown that *Torula Utilis*, fresh or dried, splits off ammonia from certain amides.

In our effort to set up a simple method for determining nicotinic acid, we have resorted to the procedure of hydrolyzing the sample with 20% sodium hydroxide solution, neutralizing to a pH of 4–5 with 60% sulfuric acid solution (keeping the volume small) and treating with acetone to precipitate the proteins, salts and practically all colored substances, the nicotinic acid remaining in solution. The acetone solution of the nicotinic acid is filtered off and evaporated. Any residual foreign coloring matter can be further reduced by treating the acetone solution before evaporation with Darco G-60 (0.2 Gm. per 100 cc.). The subsequent aqueous solutions then yield satisfactory, low-colored blanks. Using a method of this type, described in detail below, a variety of material has been assayed and the findings are shown in attached table.

METHOD

(*A*) *Reagents.*—1. Cyanogen bromide solution: Cold 10% potassium cyanide solution is added dropwise to cold saturated bromine water until the latter is just decolorized.

2. Amino reagent: Five Gm. of *p*-amino acetophenone and 10.2 cc. of concentrated hydrochloric acid (sp. gr. 1.19) are made up to 50 cc. with distilled water.

3. Twenty per cent sodium hydroxide solution (243.8 Gm. sodium hydroxide per liter).

4. Sixty per cent sulfuric acid solution (899 Gm. sulfuric acid per liter).

5. Standard nicotinic acid solution 20 μg. per cc.—prepared daily by diluting an alcoholic solution, containing 1000 μg. nicotinic acid per cc., with water.

6. Phosphate buffer solution: To 16.5 Gm. of 85% potassium hydroxide, 19.1 cc. 85% phosphoric acid and 82 cc. of *N* sodium hydroxide solution is added sufficient distilled water to make 250 cc.

7. Absolute alcohol.

8. Acetone.

(*B*) *Hydrolysis, Neutralization and Acetone Treatment.*—To the sample (this should be of the order of 0.1 to 0.5 Gm. depending upon the potency of the material being assayed) in a 125-cc. Erlenmeyer flask are added 3 cc. of 20% sodium hydroxide solution and 5 cc. of distilled water. The mixture is heated on the steam bath for one hour with occasional shaking—a glass funnel is placed in the mouth of the Erlenmeyer flask to prevent excessive loss of water by evaporation. The mixture is cooled, neutralized and adjusted to a pH of 4–5 by means of 1.0 cc. of 60% sulfuric acid. The volume is kept small and at this point certain protein material and humins (colored substances) precipitate out. After 80 cc. of acetone have been added, the contents of the flask are heated at the boiling point with agitation for a minute and then cooled to room temperature. At this point the bulk of the sodium sulfate, proteins and colored substances exist as a solid phase which is removed by suction filtration through a filter aid on paper on a Büchner funnel. The residue on the filter is washed with several 10-cc. portions of acetone. The combined acetone filtrate and washings is freed of its acetone by evaporation on the steam bath. The small residual aqueous phase is taken up in 20 cc. of distilled water[1] and transferred to a 250-cc. volumetric flask. Then 5 cc. of the phosphate buffer solution are added and the volume made up to the mark with distilled water. The pH varies from 6–6.5.

(*C*) *Opening Pyridine Ring.*—Four 25-cc. amber graduated cylinders are employed. To cylinders 3 and 4 are added, respectively, 1 cc. and 2 cc. of the standard nicotinic acid solution. To each of the four cylinders are added 10 cc.[2] of the "sample solution." To cylinders 1, 2 and 3 are added, respectively, 4 cc., 2 cc. and 1 cc. of distilled water. The cylinders are stoppered and heated in a hot water bath at 70–80° C. for 10 minutes. To each of cyl-

[1] If any precipitate or oil (fatty acids from the saponification of fats) is present, the aqueous phase is filtered through paper, washing with several 20-cc. portions of distilled water.

[2] This 10 cc. should contain 20 μg. or less of nicotinic acid. If that is not the case the "sample solution" should be diluted further.

inders 2, 3 and 4 are added 2 cc. of the cyanogen-bromide solution allowing exactly 10 seconds between the additions to the several cylinders. Each is vigorously shaken and returned to the hot water bath and heated for another 5 minutes. At the end of the 5-minute period the cylinders are removed in order at 10-second intervals and transferred to an ice water bath and chilled quickly.

(*D*) *Production of Color.*—After standing 5 minutes in the ice water bath, 0.5 cc. of the *p*-amino-acetophenone solution is added to each cylinder. The volume is now 14.5 cc. The cylinders are placed in the dark.

(*E*) *Measurement of Color.*—After standing 5 minutes in the dark the volume is made up to 25 cc. with absolute alcohol (11.2 cc. required) and the color is measured photoelectrically within the next 4 minutes in the Pfaltz and Bauer Fluorophotometer using a G. E. Mercury Lamp, Type H3, 85 Watts as the source of light. Filter glass (Jena), a combination GG-3 and BG-12 (Corning combination is 038 and 511) giving a peak at 4500 A., is placed between the solution and the light source. The iris diaphragm, which controls the amount of light admitted to the solution, is practically closed.

To set the instrument place 12.5 cc. of aqueous alcohol (14.5 cc. distilled water and absolute alcohol *q. s.* 25 cc.) in the 4-cm. cuvette; set the transmission switch at highest intensity and the variable resistance at 10, and the TrH reading on the galvanometer is then set at zero extinction by means of a shunt resistance.

Now place 12.5 cc. of aqueous alcoholic solution from each of the four cylinders individually in order into the 4-cm. glass cuvette and read the extinction directly from the galvanometer. The extinction for the blank (cylinder 1) is subtracted from the readings for the solutions in cylinders 2, 3 and 4, thereby giving values known as *A*, *B* and *C*. The nicotinic acid is calculated as follows:

$$\frac{A}{(B + C) - 2A} \times \frac{60}{1} \times \frac{25}{1} \times \frac{1}{\text{amt. of sample}}$$

(Amt. = cc., grams, capsules, tablets taken.)

In the attached table the materials tested and the potencies found are shown together with the values which, from other work, have been considered the nicotinic acid content of the substance in question. These latter values are in some instances based on bio- and microbiological assays, and, in others, on known amounts of nicotinic acid (or amide) used in making the mixtures.

SUMMARY

1. Cyano-*p*-amino-acetophenone reaction has been applied satisfactorily to the determination of nicotinic acid in pharmaceutical products.

2. By treating the alkali hydrolyzates of materials containing nicotinic acid with acetone the salts, proteins and colored substances, which interfere with photoelectric measurement of the cyano-*p*-amino-acetophenone color compound of nicotinic acid, are removed to a point where they no longer have any significant effect.

Table of Potencies

	Sample Taken or Potency Expected		Found	
Nicotinic acid	0.500	mg.	0.499	mg.
Nicotinamide	0.500	mg.	0.495	mg.
Nicotinic acid tablet	100	mg./tab.	111.7	mg./tab.
	50	mg./tab.	40	mg./tab.
Nicotinamide capsule	50	mg./cap.	56.8	mg./cap.
Yeast powder, type A	0.3–0.66	mg./Gm.	0.50	mg./Gm.
			0.53	mg./Gm.
			0.86	mg./Gm.
			0.68	mg./Gm.
			0.65	mg./Gm.
Powder containing B complex, type B	0.5	mg./Gm.	0.80	mg./Gm.
			0.70	mg./Gm.
			0.54	mg./Gm.
			0.64	mg./Gm.
			0.67	mg./Gm.
Powder containing B complex, type C	2.0–2.5	mg./Gm.	2.63	mg./Gm.
			2.38	mg./Gm.
			2.31	mg./Gm.
			2.29	mg./Gm.
Syrup containing B complex	2.2–2.5	mg./cc.	2.46	mg./cc.
			2.79	mg./cc.
			3.29	mg./cc.
Yeast concentrate, liquid	1.25–2.75	mg./cc.	1.56	mg./cc.
Mixture of B factors in capsules	5.0	mg./cap.	5.6	mg./cap.
			5.04	mg./cap.
			5.43	mg./cap.
			5.2	mg./cap.
Paste in capsules, type III	10	mg./cap.	10.8	mg./cap.
Paste in capsules, type IV	10	mg./cap.	9.85	mg./cap.
			11.83	mg./cap.
Paste in capsules, type V	20	mg./cap.	20.6	mg./cap.
Paste in capsules, type VII	5.0	mg./cap.	5.27	mg./cap.
			5.20	mg./cap.
			4.85	mg./cap.
Paste in capsules, type IX	5.0	mg./cap.	5.5	mg./cap.
			5.1	mg./cap.
			5.3	mg./cap.
Paste in capsules, type XI	5.0	mg./cap.	4.95	mg./cap.

The items listed as Paste in capsules are in all instances multivitamin mixtures with suitable diluents and vehicles in soft elastic capsules.

REFERENCES

(1) Harris and Raymond, *Biochem. J.*, 33 (1939), 2037.

(2) Kodicek, *Ibid.*, 34 (1940), 712–735.

(3) Melnick and Field, *J. Biol. Chem.*, 134 (1940), 1.

(4) Arnold, Dehreffley and Lipsuirs, *Ind. Eng. Chem., Anal. Ed.*, 13 (1941), 62.

(5) Giri and Naganna, *Indian J. Med. Research*, 29 (1941), 125.

(6) Thus, Thakur and Norris, *J. Indian Inst. Sci.*, 11A (1928), 152.

(7) Gorr, Gunther and Toagner, *Biochem. Z.*, 254 (1932), 1–4.

The Solubility of Ephedrine in Liquid Petrolatum*

By Joseph Rosin, G. K. Eger and Harry Mack

Ephedrine base is extensively used as a therapeutic agent. It is generally formulated as a solution in light liquid petrolatum, commonly called "spray" or "inhalation." Accurate knowledge of the solubility of ephedrine in this solvent is, therefore, very desirable. There is, however, very little information on this subject in the literature. Moreover, ephedrine base is available in the form of a hemihydrate and also as the anhydrous article. The U. S. P. specifications permit both the hemihydrate and the anhydrous, and some difference in the degree of solubility might be expected.

The laboratory of the AMERICAN PHARMACEUTICAL ASSOCIATION (1) has reported the solubility of ephedrine in light and heavy liquid petrolatum at 25° C. It is not, however, clear from their report what type of ephedrine they used, whether anhydrous or the hydrated, and if the latter, how much water it contained and whether or not it was dried before use in their determinations. As will be shown, the water content of the ephedrine has a profound effect on the degree of solubility.

In addition to the solubility at 25° C. we also determined the solubility at 20° C.—a temperature which is frequently met with in the stock rooms of manufacturers and retail pharmacists.

EXPERIMENTAL

The solubility determinations at 20° and 25° C. were made with different lots of ephedrine and of liquid petrolatum. The hydrated ephedrine used at 20° C. showed by titration 95.26% anhydrous ephedrine, and by toluene distillation it indicated 4.65% of water. The "anhydrous" ephedrine titrated 99.8%, and by toluene distillation showed 0.27% water. The light liquid petrolatum used in these determinations had a specific gravity of 0.845^{25}_{25}.

* Presented to the Scientific Section of the A. PH. A., Detroit meeting, 1941.

For the solubility determinations at 25° C. the hydrated ephedrine used titrated 95.12% of anhydrous ephedrine and the "anhydrous" alkaloid 99.37%. The light liquid petrolatum used in these tests had a specific gravity of 0.852, and the heavy, 0.868 at 25/25. The kinematic viscosity at 37.8° C. of the light oil was 0.183, and of the heavy, 0.438.

The solubilities at 20° C. were made as follows, except 24° which was obtained with the ephedrines and the oils used for solubilities at 25° C.

The liquid petrolatum was heated between 45° and 50° C. An excess of the ephedrine amounting to 50–100% of the assumed solubility was added, and the mixture shaken in a mechanical shaker for about 6 hours. After allowing to stand over night or longer, at room temperature, the mixture was maintained at 20° C. for 5 to 6 hours with frequent agitation. A portion of the mixture was filtered through a glass filter, and the ephedrine determined in an accurately measured volume of 10 or 15 cc. of the clear filtrate. The remainder of the mixture was again shaken at room temperature in a mechanical shaker for several hours and on the following day kept at 20° C. for 5 to 6 hours with frequent shaking, and the ephedrine again determined in a filtered portion. The results are shown in Table I.

Table I.—Solubility of Ephedrine in Light Liquid Petrolatum at 20° C.

Number of Hours of Contact	Hemihydrated Ephedrine Gm. of Anhydrous Ephedrine per 100 Cc. of Soln.	Anhydrous Ephedrine Gm. of Anhydrous Ephedrine per 100 Cc. of Soln.
48	0.87	2.26
96	0.81	2.24
24*		2.207
Av.	0.84	2.235

The solubility at 25° C. was determined in a similar manner, except that the petrolatum was not preheated. The ephedrine was added to the petrolatum at room temperature (about 25° C.), the mixture shaken at room temperature for 6 hours or longer, and before withdrawal of a portion for the determination, the mixture was maintained at 25° C. for 5 to 6 hours with frequent shaking. The solubility at 25° C. was also determined for light and heavy liquid petrolatum. The results are shown in Table II.

The extraction of the alkaloid from the oil solution was effected with a moderate excess of approximately half-normal sulfuric acid. The acid solution was alkalinized with sodium hydroxide and shaken with 6 to 8 portions of ether. The combined ether extracts were shaken with two 10-cc. portions of water to remove any sodium hydroxide, and the water washings were extracted with two 10-cc. portions of ether and the latter added to the main ether solution. The bulk of the ether was allowed to evaporate at practically room temperature, then a measured excess of twentieth-normal sulfuric acid added. The mixture was then heated gently to expel all the ether, cooled, and the excess acid titrated

Table II.—Solubility of Ephedrine in Light and Heavy Liquid Petrolatum at 25° C.

Number of Hours of Contact	Light Liquid Petrolatum: Hemihydrated Ephedrine, Gm. of Anhydrous Ephedrine per 100 Cc. of Soln.	Light Liquid Petrolatum: Anhydrous Ephedrine, Gm. of Anhydrous Ephedrine per 100 Cc. of Soln.
36	1.240	3.137
	1.248	3.111
60	1.237	3.149
	1.243	3.134
Av.	1.242	3.133

Number of Hours of Contact	Heavy Liquid Petrolatum: Hemihydrated Ephedrine, Gm. of Anhydrous Ephedrine per 100 Cc. of Soln.	Heavy Liquid Petrolatum: Anhydrous Ephedrine, Gm. of Anhydrous Ephedrine per 100 Cc. of Soln.
36	1.092	2.842
	1.118	2.855
60	1.110	2.876
	1.119	2.920
Av.	1.110	2.873

with fiftieth-normal sodium hydroxide using methyl red indicator.

In the determinations at 25° C., and also the one marked 24° in Table I, the acid solution from the extraction of the oil was saturated with sodium chloride and alkalinized with an excess of 10% ammonia water instead of sodium hydroxide. By saturating the solution with sodium chloride the complete extraction of the alkaloid was attained with four portions of ether. Also, the use of ammonia water eliminated the necessity of washing the ether with water. Any ammonia retained in the ether solution was removed by gentle suction.

The results reveal a striking effect of the 5% of water in the hydrated ephedrine on its solubility in liquid petrolatum. The ratio of the solubility of the hydrated ephedrine to the anhydrous is 1:2.67 at 20° C., and 1:2.52 at 25° C. in light liquid petrolatum. For the heavy liquid petrolatum the ratio is 1:2.58 at 25° C. At the temperatures of 20° and 25° C. substantially anhydrous ephedrine is, in round numbers, $2^1/_2$ times as soluble as the hemihydrate.

It is interesting to call attention to another curious distinction in the properties of hydrated and anhydrous ephedrine. Whereas anhydrous ephedrine melts at about 34° C., the hemihydrate melts at about 40° C.

The difference in the degree of solubility between 20° and 25° is also noteworthy. At 20°, 100 cc. of the solution hold 0.84 Gm. of ephedrine calculated as anhydrous, and at 25° 1.24 Gm.—a difference of 0.40 Gm. or a little over 30%. The difference in solubility of the anhydrous at 20° and 25° is 0.64 Gm. or a little over 20%. We observed crystallization of ephedrine to take place from a saturated solution in light liquid petrolatum prepared at 25° after the solution has stood over night during which the temperature was only a few degrees below 25° C.

The results presented in Table II indicate that the difference in the solubility at 25° C. between the light and the heavy liquid petrolatum used is only about 10%.

REFERENCE

(1) N. F. Committee Bulletin, Vol. 7, No. 10 (July 1939), 328.

Enteric Coating*

By Paul V. Maney† and Ruldoph A. Kuever‡

INTRODUCTION

Interest in the mode of administering medicine in individual doses, protected by enteric coating, is on the increase. The purpose is to deliver the medication to the intestinal tract for optimum disintegration in the duodenum or jejunum. Drugs delivered in such a manner are of value because:

1. Prolonged contact of highly irritating concentrations with the mucous membrane of the stomach is eliminated.
2. Possible interference with the digestive processes of the stomach by forming precipitates with pepsin and peptones is avoided.
3. Those that are otherwise rendered inactive by the secretions of the stomach, are delivered to the intestinal tract in their therapeutically active form.
4. Delivery of a high concentration to the desired portion of the intestinal tract is possible.
5. Recently it has been disclosed that it may be possible, by means of administering medicine with delayed action, to avoid sleep interruption.

Many drugs are at present administered in enteric form. A common list follows: sodium and potassium chloride; magnesium sulfate; potassium nitrate; ammonium nitrate; ammonium chloride; ferrous sulfate; salts of salicylic acid; methenamine, sodium biphosphate combinations; mandelic acid, ammonium chloride combinations; emetine, and bismuth compounds; aminophyllin;

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theobromine; sodium acetate; phenobarbital combinations; sulfanilamide; pancreatin; bile salts and extract of bile; ovarian substance; thyroid; digitalis; and gentian violet. In addition a number of proprietary antiseptics and anthelmintics are so administered. A number of European products so coated, for the purpose of eliminating excessive intestinal fermentation, are also available.

Increase in the number of drugs thus administered coupled with the generally unsatisfactory results obtained from coatings commonly employed led to the selection of this problem for investigation.

HISTORY

The problem of enteric coating dates back many years. A large variety of coatings has been applied with varying degrees of success. However, as pharmaceutical chemistry has progressed, the value of many of these older coatings has been disproved. At the same time new materials have been made available, but equally important is the new method of testing these coatings in the human body.

In reviewing the previous methods and types of coatings, it was found that as far back as 1884 Unna (1) published reports on the use of keratin. Scoville (2) found that formalin-gelatin possessed enteric properties of limited value. In 1915 Toplis (3) suggested stearic acid and in 1928 a report by Freeman (4) supported this work. Also in 1915, Peacock (5) reported experience with salol in the coating of pills. Wruble (6) and Hilton (7) used ammoniated solutions of shellac, with variations by the latter with combinations of salol. In 1932 Husa and Magid (8) investigated a mixture of salol, stearic acid and shellac.

The use of cetyl alcohol by Mills (9) was reported in 1937. In 1938 Worton, Kempf, Burrin and Bibbins (10) published a paper on the use of a compound coating composed of stearic acid, carnauba wax, petrolatum, powdered agar and powdered elm bark,[1] in which the agar and elm bark were incorporated as mechanical agents to effect timed disintegration. The work of Bukey and Klemme (11) in 1939 reviewed the possibilities of mastic and mastic magnesium stearic combinations.

The progress that has been made in testing their efficiency has made possible the development of more satisfactory enteric coatings.

In 1915 Toplis (3) reported a testing method consisting of applying the enteric coating to an individual dose of ipecac, the resulting delayed action being the measure by which the coating was evaluated. In 1932 Husa and Magid (8) prepared coated capsules composed of methylene blue and calcium sulfide. The effectiveness of the coating was determined as follows: If the capsule dissolved in the stomach, regurgitation of hydrogen sulfide followed. If disintegration occurred in the intestines the urine was colored blue, and there was no regurgitation. If neither occurred it was evident that the capsule did not dissolve. In 1930 Wruble (6) reported the use of a mechanical device, using artificial gastric and intestinal fluids, to test the solubility of a coating.

Recently the roentgen ray has been of great value in determining the behavior of the enteric-coated products in the gastro-intestinal tract. After radiographical experimentation Bukey and Rhodes (12), in 1935, reported wide variation in the efficiency of the commercial coatings. Testing enteric coating by means of roentgen rays was applied by Worton, Kempf, Burrin and Bibbins (10) and is now generally considered the most accurate method.

EXPERIMENTAL

After a review of literature the experimental work to be undertaken was divided as outlined below:

1. Construction of a mechanical device for testing enteric coatings.
2. Correlating results of the mechanical laboratory method with those of roentgen rays.
3. Conducting a series of tests to determine to what extent tablets, now on the market, vary in respect to the disintegrating time.
4. A study of the physiological conditions in the gastro-intestinal tract, to create a type of coating which will resist the fluids of the stomach, but disintegrate in the fluids of the intestines.
5. Development of a new enteric coating.

[1] Patent numbers 2,011,586 and 2,011,587.

Construction of a Mechanical Device for Testing Enteric Coatings.—In the construction of the mechanical apparatus for laboratory testing (Figs. 1, 2 and 3), the objective was to simulate, as nearly as possible, the condition found within the gastro-intestinal tract. A brief outline of the course the tablet took when tested will describe the function of the apparatus.

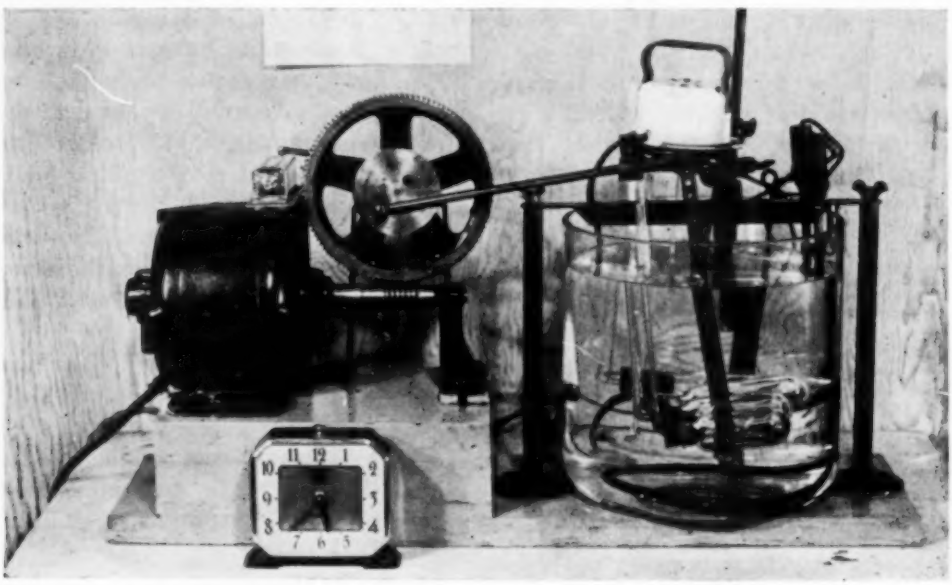

Fig. 1.—Mechanical Laboratory Apparatus for Testing Enteric Coatings.

The tablets tested were placed within an elongated tube containing the digestive fluids. The tube, having an outlet for gas escape, was placed in a rack that could swing through an arc of one hundred and twenty degrees. The motion was timed to approximately sixteen oscillations per minute. The exact number of oscillations was recorded on an automatic counter. The tablets were moved slightly by the motion and gently washed by the digestive fluids. The entire rack, containing the tubes, was immersed in a constant temperature bath maintained at 37° C. The gastric fluid employed was that recommended by Toplis and later used by Wruble (6), and Worton, Kempf, Burrin and Bibbins (10).

Artificial Gastric Fluid

Sodium chloride	1.400 Gm.
Potassium chloride	0.500 Gm.
Calcium chloride	0.060 Gm.
Hydrochloric acid 36%	6.944 Gm.
Pepsin, U. S. P.	3.200 Gm.
Distilled water, to make	1000.000 cc.

Gastric fluid made by this formula has a pH index of 1.6 which corresponds to that outlined in the text of Best and Taylor (13). To substantiate the action of the artificial gastric fluid, several tests were conducted in which the gastric juice of freshly killed hogs was used. The gastric juice from the hog, with a pH index 3, was found to have identical effects on the coating with that of the artificial gastric fluid.

In selecting an artificial intestinal fluid, the formula previously used by other workers was studied. It consists of the following and was previously used in the Pharmacopœia as a test for pancreatin:

Pancreatin, U. S. P.	2.8 Gm.
Sodium bicarbonate	15.0 Gm.
Distilled water, to make	1000.0 cc.

Fig. 2.—Tablets with New Enteric Coating after Ten Hours in Artificial Gastric Fluid. No Evidence of Disintegration Visible.

Fig. 3.—The Same Tablets as in Fig. 2, Thirty Minutes after Being Placed in Artificial Intestinal Fluid. Disintegration Is Taking Place.

Verźar and Kúthy (14), Bodansky (15), as well as others, have demonstrated that the small intestinal tract is not always alkaline even in normal cases. Karr and Abbott (16) carried out a series of experiments in this connection finding that in fasting men a gradient of pH from slight acidity in the duodenum to a slight alkalinity in the ilium was observed. With this evidence it was not felt that the alkalinity alone of the intestines would suffice. Artificial intestinal fluids were therefore made by the following formulas:

Artificial Intestinal Fluid—Alkaline

Pancreatin, U. S. P.	2.8 Gm.
Sodium bicarbonate	15.0 Gm.
Distilled water, to make	1000.0 cc.

Artificial Intestinal Fluid—Neutral

Pancreatin, U. S. P.	2.8 Gm.
Distilled water, to make	1000.0 cc.

Artificial Intestinal Fluid—Acid

Pancreatin, U. S. P.	2.8 Gm.
Hydrochloric acid to a pH index of	6.8
Distilled water, to make	1000.0 cc.

Artificial intestinal fluids, made according to the formula in Table II, were found to be more effective in disintegrating the enteric coating when one-half of one per cent of bile salts, a natural intestinal tract constituent, was added.

Correlation of Results of the Mechanical Laboratory Method with Those of Roentgen Rays.—To compare the results of the tests *in vitro* with those *in vivo*, 185 mechanical, roentgenogram, roentgenoscopic observations were recorded. The coating was developed and tested *in vitro* before being tested *in vivo*.

To determine the position of the stomach a portion of barium meal was administered and a roentgenogram taken. Discrepancies were disclosed in the two methods, the difficulty arising from the many variables in the gastro-intestinal tract. However, generally speaking, the data obtained from tests *in vitro* made possible a dependable predication of the results *in vivo*.

In vivo experiments proved that an efficient coating must be capable of withstanding artificial gastric fluid for at least ten hours *in vitro*. When transferred to the artificial intestinal fluid disintegration should take place within three hours whether it be alkaline, neutral or slightly acidic.

A Series of Tests to Determine to What Extent Enterically Coated Tablets Now on the Market Vary in Respect to the Disintegrating Time.—To determine the variation in the disintegrating time of enteric coatings now on the market, 31 tablet specimens were tested. The tabulation of results is shown in Table I.

Table I.—Variation in Disintegrating Time of Tablets

	Artificial Gastric Fluid								Artificial Intestinal Fluid					
Specimen	½	1	2	3	4	5	6	←Hours→	½	1	1½	2	3	24
1	5	5	5	0	0	0	0		0	0	0	0	0	0
2	5	5	5	5	5	5	5		5	5	5	4	3	0
3	5	5	3	0	0	0	0		0	0	0	0	0	0
4	5	5	4	3	3	3	0		0	0	0	0	0	0
5	5	5	5	4	0	0	0		0	0	0	0	0	0
6	5	5	5	4	2	2	2		2	2	2	1	1	0
7	5	5	5	5	4	0	0		0	0	0	0	0	0
8	5	5	5	5	5	4	0		0	0	0	0	0	0
9	5	5	5	5	5	5	5		5	0	0	0	0	0
10	5	5	5	5	5	5	5		5	4	1	0	0	0
11	5	5	5	5	5	5	5		5	4	3	0	0	0
12	5	5	5	5	5	4	0		0	0	0	0	0	0
13	5	5	5	5	5	5	4		3	0	0	0	0	0
14	5	5	5	0	0	0	0		0	0	0	0	0	0
15	5	5	5	5	5	5	5		5	0	0	0	0	0
16	5	5	5	5	5	5	4		4	4	3	2	2	2
17	5	5	4	0	0	0	0		0	0	0	0	0	0
18	5	5	5	4	3	3	3		3	2	1	1	1	1
19	5	4	1	0	0	0	0		0	0	0	0	0	0
20	5	5	4	0	0	0	0		0	0	0	0	0	0
21	5	5	5	4	0	0	0		0	0	0	0	0	0
22	5	5	5	4	3	0	0		0	0	0	0	0	0
23	5	5	5	4	0	0	0		0	0	0	0	0	0
24	5	5	5	5	5	5	5		5	4	0	0	0	0
25	5	5	3	2	0	0	0		0	0	0	0	0	0
26	5	5	5	5	5	5	5		5	5	5	5	5	5
27	5	5	4	0	0	0	0		0	0	0	0	0	0
28	5	5	5	5	5	5	4		4	0	0	0	0	0
29	5	5	5	4	0	0	0		0	0	0	0	0	0
30	5	5	5	3	3	3	3		3	1	0	0	0	0
31	5	5	5	5	5	4	0		0	0	0	0	0	0

Five tablets were used in each test. The figures indicate the number of tablets *not* disintegrated at the time designated. Specimen No. 19 was nearly disintegrated in the gastric fluid in one and one-half hours, while in contrast specimen No. 16 remained in the gastric fluid and likewise in the artificial intestinal fluid for twenty-four hours with only three disintegrating.

The method of testing was the same as that described under mechanical testing apparatus. The tablets were placed in the artificial gastric fluid and

permitted to remain there, with the machine in motion, for six hours. They were then treated in like manner with the alkaline intestinal fluid. Five tablets were used in each test. Tablets of the same source but of different composition were treated simultaneously to test the same coating on different tablets under identical conditions. Two separate tests were made on each specimen. Little variation was found; therefore, only one result was tabulated.

A Study of the Physiological Conditions in the Gastro-Intestinal Tract, to Determine the Proper Type of Coating Which Will Resist the Fluids of the Stomach, but Disintegrate with the Fluids of the Intestines.—The selection of suitable material for enteric coating necessitated a study of the physiological aspect of the gastro-intestinal tract.

Bukey and Brew (17) observed that human stomachs emptied quickly on high carbohydrates, more slowly on high protein and still more slowly on high fat content diets.

The time of day when tablets are administered has an influence on the rate of discharge from the stomach, the tablets remaining in the stomach longer as the day progresses. However, roentgenoscopic examinations show that tablets administered on an empty stomach early in the morning may remain there for as long as nine hours.

The individual who exercises regularly was, as a rule, found to discharge the stomach contents more rapidly than the individual who does not (18).

Bukey and Brew (17) found that the size of tablet did not alter the rate with which the stomach emptied. But the work of Crane and Wruble (19) indicated that capsules, as a rule, passed more rapidly from the stomach than did tablets.

An interesting observation is that when several tablets are swallowed simultaneously some may pass into the intestines while others may remain in the stomach for several hours (Fig. 4).

Worton, Kempf, Burrin and Bibbins (10), citing Bukey and Brew (17) and Briggs (20), pointed out that an efficient enteric coating should be stable for about six hours and then disintegrate quickly, regardless of the pH of the body fluid surrounding it. However, the recent work of Crane and Wruble (19), after investigating enteric coatings by means of 1000 roentgenograms and roentgenoscopic observations, indicated that approximately 15 per cent of the tablets and capsules remained in the stomach for nine to ten hours.

The optimum position for disintegration, as outlined by Bodansky (15), is in the duodenum or jejunum. As these portions of the small intestines are best adapted for absorption it can be seen that the most satisfactory coating should disintegrate immediately after passing from the stomach.

The pH index of the duodenal contents varies after the ingestion of certain types of food substances according to McClure, Montague and Campbell (21). Duodenal contents were acid after ingestion of high protein diets, and alkaline after ingestion of high fat and carbohydrate food substances. No relation was found between the pH of the duodenal contents and the stimulation of the flow of bile and pancreatic juice.

The gastric juice is the first principal medium to which the coating is exposed, and according to Best and Taylor (13) the gastric juice is composed essentially of lipase, pepsin and hydrochloric acid with rennin in cases of infancy. According to Harrow and Sherwin (22) acid chyme ejected from the stomach comes in contact with the intestinal juice, bile and pancreatic juice.

The work of Verźar and Kúthy (23) indicates that very little digestive action on fatty acids takes place in the gastric juice under normal conditions. This work is supported by Harrow and Sherwin (22). However, Verźar and Kúthy reported that the alkaline soaps of the fatty acids are soluble at a pH index higher than is normally found in the human intestine. They also demonstrated that fatty acids,

Fig. 4.—A Photograph of a Roentgenogram Two Hours after Administration of Six Tablets. Two Tablets Are in the Stomach, and Four Have Passed into the Intestines.

finely emulsified, form a diffusible suspension with paired salts of sodium taurocholate and sodium glycocholate. Such suspensions are formed in neutral or slightly acidic medium and have a low surface tension. They postulate the formation of fatty acid suspensions by means of bile salts complexes. This leads to a new principle of enteric coating which has been developed and demonstrated. It was possible to prepare a coating of suitable fatty acids, properly modified, which will resist the action of the gastric juice for a period of ten hours. Tablets coated with this material will

not disintegrate while they remain in the stomach. Upon passing into the intestinal tract disintegration will occur quickly irrespective of whether the fluid is alkaline, slightly acidic or neutral.

Development of a New Enteric Coating.—The use of shellac and shellac combinations has been recommended. A series of tests revealed that over a period of two years tablets so coated increased their disintegrating time materially. Salol and karatin used for a long time were found unreliable, thus supporting the information in "Remington's Practice of Pharmacy" (24) and Scoville's "The Art of Compounding" (25). Good results were obtained in some cases with cetyl alcohol. These coatings were tested *in vitro*.

The fatty acids investigated were lauric, myristic, stearic and eutectic mixtures of palmitic and stearic. The individual acids as well as many mixtures were investigated. It was found desirable to produce a coating with greater firmness and elasticity than the acids themselves could supply. The addition of opal wax[1] and castor oil was found to be of value. A small quantity of cholesterol increased the ease with which the coating would emulsify. When tested *in vitro* and by roentgen rays, this coating gave favorable results. However, in some cases disintegration did not take place until the tablets reached the colon. To overcome this, sodium taurocholate, a timing factor, was included. The formula of the completed coating follows:

New Enteric Coating

Myristic acid	68%
Opal Wax	25%
Castor Oil	2%
Cholesterol	1%
Sodium taurocholate	4%

For test purposes barium sulfate tablets, which would readily disintegrate when brought in contact with the digestive fluids, were used. The coating is applied as follows: Melt the myristic acid, opal wax, castor oil and cholesterol and dilute these with an equal volume of a mixture of equal parts of ethylene dichloride and benzin. To this mixture add the sodium taurocholate previously dissolved in ten times its own weight of absolute ethyl alcohol.

This coating, kept warm at 50° C., may be applied in the ordinary manner using special precaution to build a coating of uniform thickness. No dusting powder is necessary. Hot and cold air are desirable to evaporate the solvent and harden the application. A number of applications are necessary to bring the coating up to the desired thickness.

Tablets so coated were found to remain in the stomach in certain individuals for six hours, but disintegrated within two hours after entering the duodenum. In other cases, the tablets left the stomach in two hours and disintegrated within thirty minutes in the small intestines. All tests indicated that the coating possessed highly efficient enteric properties.

For results of tests conducted in the mechanical laboratory apparatus see Table II. For roentgeno-

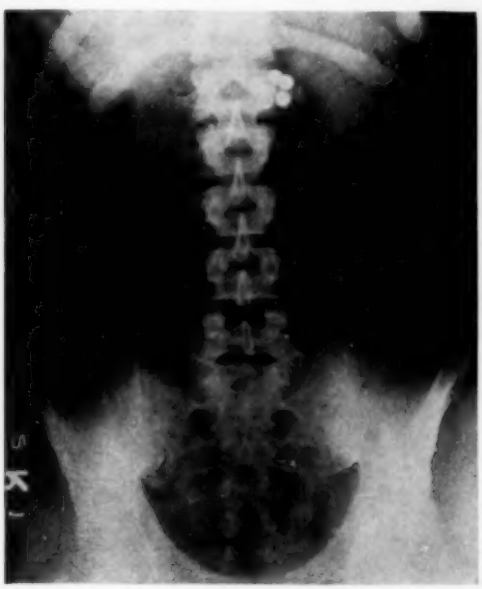

Fig. 5.—Photograph of a Roentgenogram, Five Minutes after Administration of Six Tablets Protected by the Developed Enteric Coating. All Tablets Are in the Stomach.

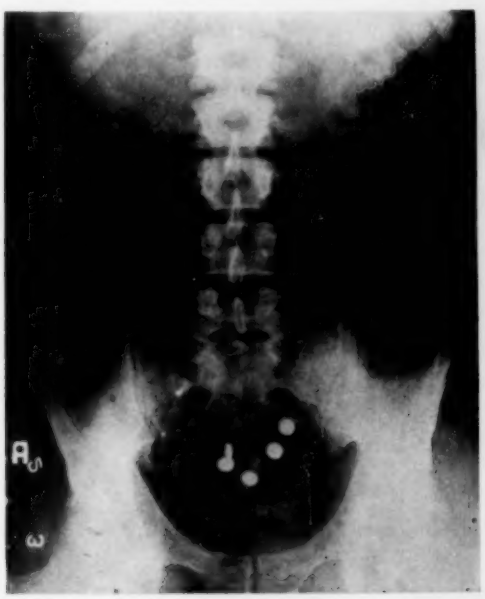

Fig. 6.—A Photograph of a Roentgenogram of the Same Individual as Is Shown in Fig. 5, but Taken Four Hours Later. All of the Tablets Are Now in the Intestines.

[1] Commercial name for hydrogenated castor oil, E. I. du Pont de Nemours & Co.

Fig. 7.—A Photograph of a Roentgenogram of the Same Individual as Is Shown in Figs. 5 and 6, but Taken Six Hours Later. All Tablets Have Disintegrated or Are in That Process.

grams showing results of the same coating see Figs. 5, 6 and 7.

Table II.—Tests in Mechanical Laboratory Apparatus

Five Tablets in Artificial Gastric Fluid Without Disintegration	Time Required for Disintegration in Artificial Intestinal Fluid
2 hours	4 hours in intestinal, neutral[a]
2 hours	5 hours in intestinal, acid[a]
2 hours	1/2 hour in intestinal, alkaline[a]
4 hours	3 hours in intestinal, neutral
4 hours	4 hours in intestinal, acid
4 hours	1/2 hour in intestinal, alkaline
6 hours	2 hours in intestinal, neutral
6 hours	3 hours in intestinal, acid
6 hours	1/2 hour in intestinal, alkaline
8 hours	2 hours in intestinal, neutral
8 hours	2 hours in intestinal, acid
8 hours	1/2 hour in intestinal, alkaline
10 hours	1 1/2 hours in intestinal, neutral
10 hours	2 hours in intestinal, acid
10 hours	1/2 hour in intestinal, alkaline

[a] See formulas for artificial intestinal fluid.

SUMMARY

A satisfactory method to determine the efficiency of enteric coatings, *in vitro*, has been developed.

In vitro results of tablets tested compare favorably with those obtained by roentgen rays *in vivo*.

Wide variation with respect to the disintegrating time was found in those tablets now on the market.

The rate with which the stomach empties and the fact that the alkalinity of the intestinal fluid cannot be relied on are important factors.

An enteric coating has been developed that demonstrated a high degree of efficiency.

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"The success of a discovery depends upon the time of its appearance."—Weir Mitchell